

4704

STUDIES ON STARCH-DEGRADING ENZYMES

by

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PREFACE

I wish to thank Dr. C.T. Greenwood for his constant advice and encouragement throughout the period of this research. Some of the work in Sections 4 and 5 has been published in conjunction with Drs. C.T. Greenwood and A.W. MacGregor, and reprints are inserted at the end of the thesis.

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General Introduction

Starch has been the subject of many investigations and the essential features of its structure and physicochemical properties are well established [Greenwood (1956 and 1964 a), Whelan (1958)]. The polysaccharide is now known to consist of at least two components, amylose and amylopectin, which may be separated by a variety of methods [Schoch (1945), Muetgeert (1961)]. Amylose is an essentially linear glucan, in which the units are joined by α -1:4 linkages, whilst amylopectin contains, in addition, 4-5% of α -1:6 glucosidic branch-points.

Many techniques are available for investigating the properties of starch but, of these, enzymic methods are particularly important for studying problems of fine structure. Several important starch-metabolising enzymes occur in plants, where they are often found in association with α -amylase, a carbohydrase which is not easily removed from preparations of other enzymes. While an investigation of the action-patterns of α -amylases is of intrinsic interest, it is important to study their properties, so that methods may be devised to deactivate the amylases preferentially and thus to facilitate the purification of other carbohydrases.

With the exception of malted barley α -amylase, plant α -amylases have not been extensively investigated. The

work in this thesis is an attempt to study the properties and action-patterns of several cereal and bean α -amylases.

In the first section a brief account is given of the action of the more important types of starch-degrading enzymes. The second section describes the general experimental techniques used in this work.

The extraction and purification of plant α -amylases, are discussed in Section 3. The method of preparation involves acetone fractionation, heat-treatment and glycogen-complex formation, and the purified amylases are shown to be free from other starch-metabolising enzymes.

Section 4 describes studies of the general properties of the α -amylases from oats, rye and wheat. The effect of pH and various reagents on the activity of these cereal enzymes and of the α -amylases from soya- and broad-beans has enabled the nature of the amino acids at the enzyme active centres to be investigated.

Qualitative and quantitative investigations of the action-patterns of bean, cereal, bacterial and mammalian α -amylases are presented in the last section. A description is given of kinetic studies of the initial stages of amylose-degradation by the amylases from oats, rye and wheat. The actions of α -amylases from dormant and germinated cereals are compared, and contrasted with those of the enzymes from soya- and broad-beans, B. subtilis and porcine pancreas. Theories are proposed for the

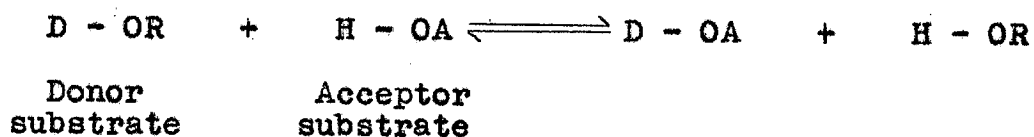
action-patterns of different types of α -amylases and hypothetical schemes for the enzyme active centres are discussed.

Section 1

The Enzymic Degradation of Starch

The Enzymic Degradation of Starch

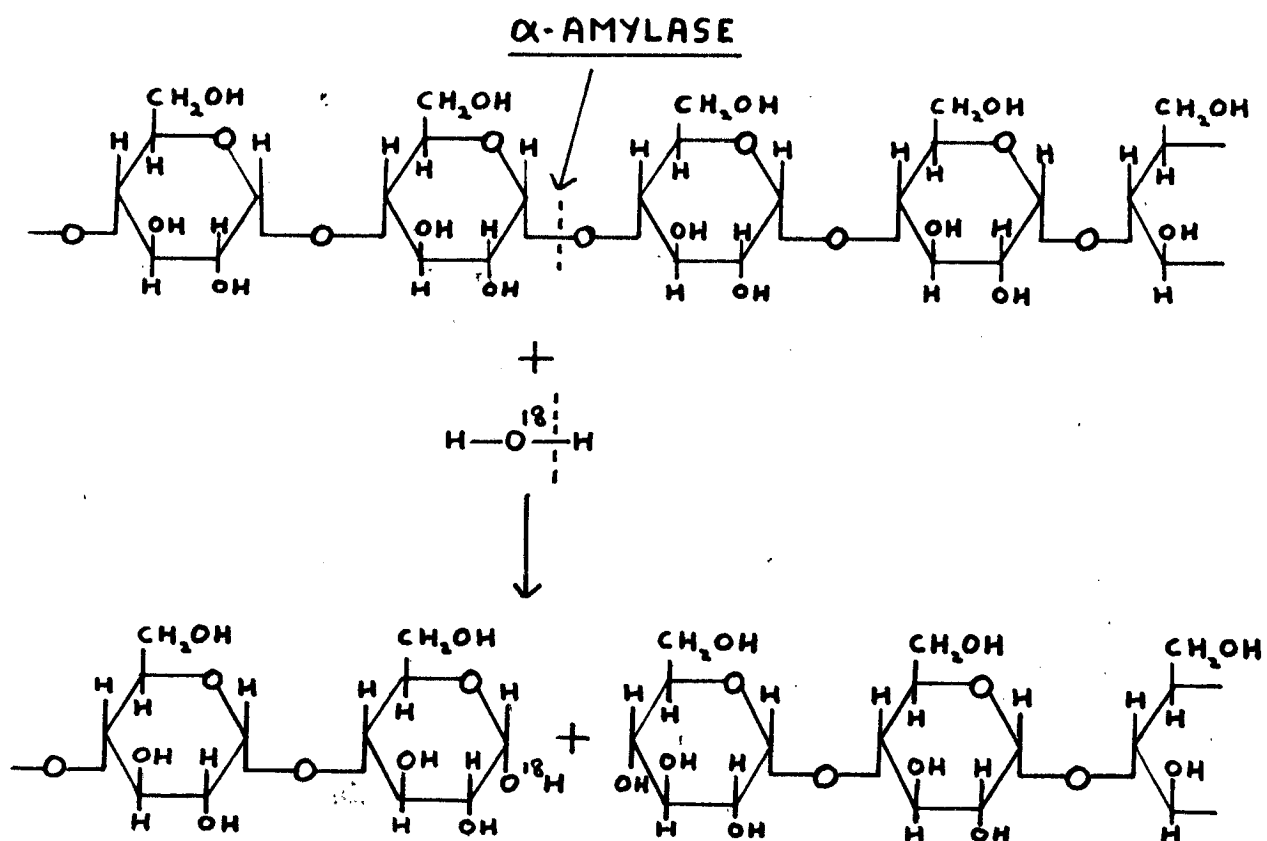
The reactions of starch-metabolising enzymes may be represented by the following general equation



The group OR of the donor substrate is removed during the reaction, and is replaced by group OA of the acceptor. An example of such a reaction is shown in Fig. 1.1, which illustrates the action of α -amylase on starch or glycogen [Mayer and Larner (1959), Halpern and Leibowitz (1959)]. The polysaccharide is the donor substrate, D - OR, and the acceptor is water. The enzyme splits the α -1:4 bond at the CI position. It has been shown for a number of carbohydrases e.g. muscle phosphorylase [Cohn (1949)], β -amylase, porcine pancreatic and Bacillus subtilis α -amylases [Mayer and Larner (1959)] that fission occurs to yield a glucosyl (D-) and not a glucosidic (DO-) grouping. For the amylases, this was done by using H - O¹⁸H as acceptor substrate and demonstrating that the heavy oxygen isotope was contained in the products of type D - OA and not H - OR. Different groups of starch-degrading enzymes may be distinguished on the basis of the position of equilibrium attained in the reaction. While D- enzyme and phosphorylase can degrade starch, their reactions are freely reversible and, under

FIG. 1.1

α -amylolysis of α -1:4 glucans



suitable conditions, these enzymes may be used to synthesise amylose-type material. The depolymerisation catalysed by hydrolases, however, is generally considered to be irreversible, although Abdullah and French (1966) have claimed that the reaction of β -amylase may be reversed to produce maltotetrose from maltose.

A brief description of the main types of starch-degrading enzymes is given below.

α -Amylases. These are endoenzymes, which fragment amylose and amylopectin molecules by hydrolysis of α -1:4 bonds other than those at the glucosidic chain-ends. The products formed have the reducing group in the α -configuration [Kuhn (1925)], hence the name of the enzymes. The sugars produced from amylose are linear oligosaccharides. High molecular-weight branched dextrans are obtained, however, from amylopectin and glycogen, for the α -1:6 links are resistant to α -amylolytic attack. The preparation, properties and action-patterns of α -amylases are discussed further in Sections 3, 4 and 5.

β -Amylase. The enzyme attacks the non-reducing ends of glucosidic chains in amylose, amylopectin and glycogen, hydrolysing alternate α -1:4 bonds to produce β -maltose. It is one of the few carbohydrases known which cause inversion of configuration at the newly-formed reducing groups. As β -amylase is unable to hydrolyse α -1:6 linkages, large branched dextrans are produced by its action on amylopectin and glycogen. Many amylose samples

are incompletely converted to maltose by β -amylolysis, but the nature of the anomaly which halts the enzymic action is not yet known.

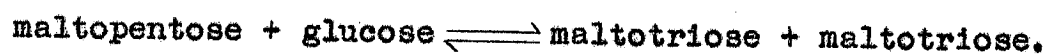
There are three mechanisms by which β -amylolytic attack may take place: (1) a single-chain mechanism in which the enzyme, having formed a complex with a substrate molecule, hydrolyses that molecule completely before attacking a second molecule of substrate; (2) a multi-chain mechanism in which the enzyme acts randomly on all substrate molecules, or (3) a "multiple attack" mechanism in which the enzyme splits off several maltose molecules per encounter with the substrate molecule, and then diffuses away to combine with another molecule of substrate.

Investigations of the β -amylolysis of small substrate molecules (DP<50) seem to suggest that the action takes place by "multiple attack" [Bailey and Whelan (1957), Bailey and French (1957)]. Although conflicting evidence has been presented for the mechanism of attack by β -amylase on large substrate molecules, the results of recent workers appear to favour the multichain mechanism [Husemann et al. (1964), MacGregor (1964)].

D-glucose-producing amylases [Manners (1962)]. These are enzymes, found in moulds and bacteria, which attack amylose and amylopectin at the non-reducing chain-ends, to liberate D-glucose in a step-wise manner.

D-enzyme [Peat, Whelan and Rees (1953 and 1956), Peat, Whelan and Kroll (1956)]. The enzyme is a transglycosylase,

which transfers two or more glucose units from a maltodextrin to a suitable acceptor (D-glucose or saccharides, degree of polymerisation ≥ 3), forming only α -1:4 glucosidic bonds. Although D-enzyme is generally considered as a starch-synthetase, degradation occurs and the average degree of polymerisation of a mixture of saccharides decreases, if glucose is added to the D-enzyme-saccharide digest, e.g.



Walker and Whelan (1957) showed, by use of radioactive substrates, that glucose is incorporated into the reducing ends of newly-formed shorter dextrans.

Phosphorylase [Whelan (1955)]. This enzyme also is capable of catalysing the synthesis of polysaccharide but, in the presence of amylose and an excess of inorganic phosphate, it may cause polymer degradation. α -Glucosyl groups are transferred from the non-reducing end of an amylose chain to inorganic phosphate, yielding glucose-1-phosphate. There are similarities between the actions of phosphorylase and β -amylase, for the degradation of a linear substrate by either enzyme may be halted by a structural anomaly in the substrate molecule.

R-enzyme [Hobson et al. (1951), Peat et al. (1954)].

R-enzyme hydrolyses the α -1:6 linkages of amylopectin and amylopectin β -limit dextrin, but has little effect on glycogen. Steric hindrance, due to the compact nature of the glycogen molecule, is thought to impede the enzymic

action. The enzyme can remove small side chains of two to four glucose units, joined by an α -1:6 bond to a base chain of α -1:4-linked glucosidic units [Wild (1954)]. It does not, however, hydrolyse isomaltose nor remove single glucose residues joined by α -1:6 linkages to maltodextrin molecules [Roberts (1953)]. MacWilliam (1958) and MacWilliam and Harris (1959) claim to have shown that the R-enzymes of broad-beans and malted barley may be fractionated into two components, one which acts on amylopectin, and the other which attacks small branched dextrans obtained by the salivary α -amylolysis of amylopectin.

Isoamylase [Gunja et al. (1961)]. Isoamylase is very similar in action to R-enzyme, but it hydrolyses the outer α -1:6 linkages of both amylopectin and glycogen.

Amylo-1:6-glucosidase [Cori and Lerner (1951)]. This enzyme removes terminal α -glucose units linked by α -1:6 bonds to primary hydroxyl groups of the muscle-phosphorylase-limit dextrans of amylopectin or glycogen.

Section 2

General Experimental Techniques

2a.

Determination of Enzyme Activity(1) α -Amylase Activity

In the preparation of α -amylases from soya-beans and cereals, a method of measuring α -amylase activity is required which is unaffected by the presence of β -amylase.

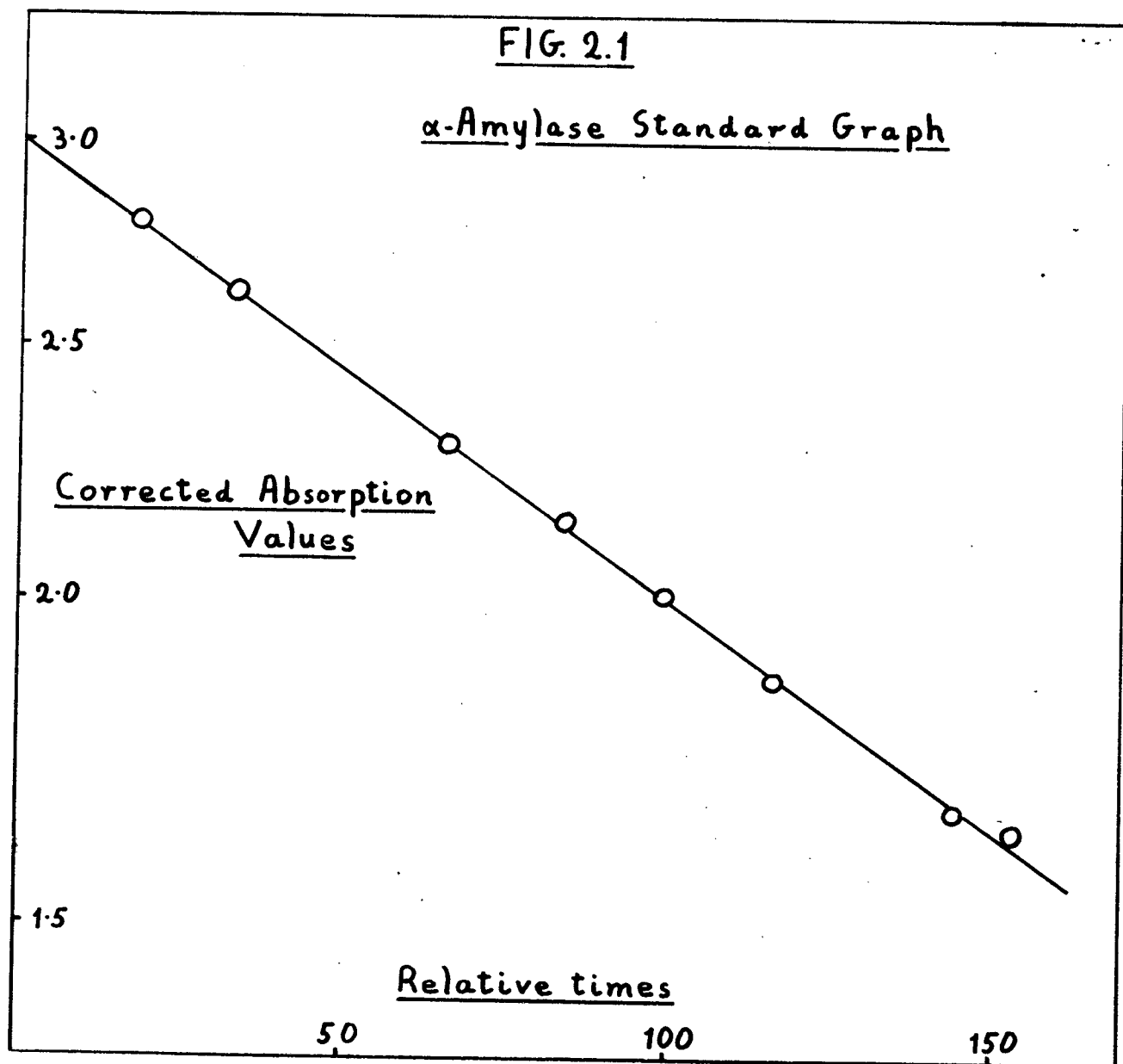
A modification of the Briggs procedure (1961) was used, with amylopectin β -limit dextrin as the substrate. With such a substrate, the initial decrease in the ability of the dextrin to stain with iodine gives a measure of the α -amylase activity, even in the presence of β -amylase. Although the first α -amylolysis will be followed by β -amylolysis of the liberated non-reducing end groups, it was shown that the action of the β -amylase had negligible effect on the iodine-staining properties of the partially-degraded dextrin.

In this modified method, the unit of activity (iodine-dextrin-colour unit) is defined as that amount of enzyme decreasing the iodine-stain of a standard dextrin digest by one third in 100 minutes. It thus follows that a quantity of enzyme which decreases the iodine-stain by one third in, say, one minute must be equivalent to 100 units of activity.

Since it is not always possible to remove a sample from a digest when the iodine-dextrin absorption value has fallen by exactly one third, enzymic activities may be calculated from absorption values of samples taken at other times, by the use of a standard graph (Fig. 2.1.). This graph was constructed (using digests containing different

FIG. 2.1

α -Amylase Standard Graph



dilutions of salivary α -amylase) in such a way that the absorption value of the dextrin-iodine complex decreased from 3.00 to 2.00 in 100 minutes. It has been shown that, in the range given in Fig. 2.1, the curves of absorption value versus time are very similar for salivary and plant α -amylases.

In practice, unless otherwise stated, digests were prepared as follows: acetate buffer (1 ml.; 0.2 M; pH 5.5), amylopectin β -limit dextrin solution (1-2 ml.; 6 mg. dextrin) and enzyme solution in a total volume of 8 ml..

The digests were incubated at 35°C and, at appropriate intervals, 2 ml. samples were removed and added to iodine solution (1 ml.; 0.2% iodine in 2% potassium iodide) and hydrochloric acid (0.2 ml.; 5 N). The volume was made up to 50 ml. with distilled water and the absorption value of the solution was measured at 540 m μ , using an "EEL" photoelectric colorimeter (Yellow-green filter No. 625).

Each absorption value was then multiplied by (3.00/absorption value at time = 0) to give the "corrected absorption value", and from this and the standard graph, the "relative time" for each reading was obtained. The "relative time" divided by the actual time of sampling the digest gave the α -amylolytic activity in iodine-dextrin-colour units.

The specific activity of an α -amylase solution was calculated by dividing the enzymic activity by the protein concentration (mg./ml.) in the digest.

(11) β -amylase activity

Digests contained freshly-prepared soluble starch solution ("Analar", B.D.H.; 15 ml.; 0.6%), acetate buffer (2 ml.; 0.2 M; pH 3.6) and sufficient enzyme (0.1-1.0 ml.) to produce 2-3 mg./ml. of maltose in the digest under the experimental conditions. After incubation of the digests at 35°C for 30 minutes, 1 ml. samples were withdrawn and their maltose content determined by the alkaline ferricyanide method of Lampitt et al. (1955). (See Section 2 d.)

The β -amylase activities are given as mg. of maltose produced per mg. of protein per ml. of digest.

2b. Estimation of Protein Concentration

The routine determination of the protein content of solutions was carried out by measuring the optical density (O.D.) of the solutions at 280 m μ in an SP500 U.V. and visible spectrophotometer. For purification studies, the O.D. of a 0.1% solution in a 1 cm. cell was taken as 1.0.

2c. Preparation of Substrates

Starch was extracted from potatoes as described by Banks, Greenwood and Thomson (1959).

Linear amylose was obtained by leaching potato starch granules at 60°C [Banks, Greenwood and Thomson (1959)]. A 0.5% aqueous suspension of the granules - which had been pre-treated by boiling under reflux with 80% methanol for 2 hours - was stirred in a nitrogen atmosphere at 60°C for 15 minutes.

The mixture was quickly cooled to room temperature and the swollen granules were removed by centrifugation. The supernatant was passed through a grade 3 sintered filter, then linear amylose was precipitated from the solution, and subsequently recrystallised, as the butanol complex.

A conventional dispersion of potato starch at 100°C was used to prepare non-linear amylose and amylopectin [Banks, Greenwood and Thomson (1959)]. Starch was added to boiling de-oxygenated water in a nitrogen atmosphere to give a 0.5% solution. After boiling under nitrogen, with stirring, for 30 minutes, the mixture was allowed to cool to 60°C and thymol (1 g./litre) was added. On standing at room temperature for 72 hours, the amylose-thymol complex precipitated from the solution and was removed on a Sharples super-centrifuge. The product was recrystallised as the butanol complex.

When the amylose had been removed, the supernatant liquid was shaken with ether to extract excess thymol, the ether then being distilled off on a rotatory evaporator at <40°C. Amylopectin was obtained by freeze-drying the remaining aqueous solution.

The β -limit dextrin was prepared from this amylopectin by digesting the substrate (0.6% aqueous solution) for 48 hours at 35°C with crystalline sweet-potato β -amylase (Worthington Biochemical Corporation, New Jersey, U.S.A.). The enzyme was then deactivated by heating the mixture for 5 minutes on a boiling-water bath, and the digest was cooled and filtered. Finally the solution was concentrated on a rotatory evaporator to give a dextrin content of 5-6 mg./ml..

Linear maltodextrins were prepared by the paper-chromatographic separation (see Section 2f.) of the products from an α -amylolysis of amylose.

2d.

Estimation of Reducing Sugar
and Polysaccharide Concentration

Glucose and maltose were estimated directly by the alkaline ferricyanide method of Lampitt et al. (1945). The concentrations of oligo- and polysaccharide solutions were determined by hydrolysing the saccharides to glucose [1.5 N sulphuric acid for 2 hours at 100°C, Pirt and Whelan (1951)] and subsequent measurement of the reducing power by the ferricyanide method.

The hydrolysis conditions given above were shown to be satisfactory for the degradation of both oligosaccharides and polysaccharides to glucose.

2e.

Viscosity

The procedure used is that described by Greenwood (1964).

Viscosities were measured in modified Ubbelohde viscometers [Ubbelohde (1937), Davis and Elliot (1949)], immersed in a water bath at $25 \pm 0.01^\circ\text{C}$. All solvents and solutions were filtered through G4 sintered-glass filters before use.

A measured volume of filtered solution was pipetted into the viscometer, aliquots of pure filtered solvent were added and the flow-time for each dilution was determined to 0.1 seconds by means of a stop-watch. The solvent flow-time was measured on a separate run.

The concentration, c , of the initial filtered solution was measured by the alkaline ferricyanide method (see Section 2d.), and expressed in g./ml..

In capillary viscometers of the type used, the viscosity, η , is given by

$$\eta = A\epsilon t - \frac{B\rho}{t}$$

where A and B are constants, t is the flow-time of a fixed volume and ρ is the density of the liquid.

B is the kinetic-energy factor arising from the finite velocity with which the liquid leaves the capillary. By the use of standard liquids, the value of B for the viscometers used has been shown to be negligible [Banks (1960)].

Thus the specific viscosity, η_{sp} , of a polymer is given by

$$\eta_{sp} = \frac{\eta_s - \eta_o}{\eta_o} = \frac{\rho_s t_s - \rho_o t_o}{\rho_o t_o}$$

where the subscripts s and o refer to the solution and solvent respectively.

For dilute polymer solutions it may be assumed that $\rho_s = \rho_o$, and thus $\eta_{sp} = \frac{t_s - t_o}{t_o}$.

Values of the limiting viscosity number, $[\eta]$, were then determined from graphs of $\frac{\eta_{sp}}{c}$ versus c , as $[\eta] = \lim_{c \rightarrow 0} \frac{\eta_{sp}}{c}$.

2f.

Paper chromatography

Qualitative separations of oligosaccharide mixtures were carried out on Whatman No. 1 chromatography paper, using the multiple-ascent technique with n-propanol:water (70:30 v/v) as

the solvent [French^{et al.}(1965)] at 20°C. Chromatograms were developed by the method of Trevelyan et al. (1950), and maltodextrins were identified by comparison of the distances travelled by the unknown sugars with those travelled by standard samples kindly donated by Professor W.J. Whelan.

For a quantitative investigation, a sample of the digest was evaporated to a small volume on a rotatory evaporator, and the syrup was streaked onto 3MM chromatographic paper. Standard sugars were also applied to the chromatogram, and separation of the maltodextrins was carried out as above. The section containing the standards was then cut off and developed, and on this basis the remainder of the chromatogram was cut into sections, each section corresponding to one oligosaccharide. Sugars were removed from the paper by irrigation with water; recovery of oligosaccharides from the chromatogram was shown to be 97-100%.

Section 3

Preparation of the α -Amylases

3.

Introduction

When extracted from living cells, enzymes are generally obtained in complex mixtures. Thus an enzyme must be purified and shown to be free of other enzymes capable of attacking the same substrate, before its properties can be studied.

Several methods of purification have been used to prepare α -amylases. These have often involved fractionation by the addition of salt or an organic solvent.

In 1947, Meyer et al. described the isolation and, the first crystallisation of an α -amylase. The enzyme (from porcine pancreas) was purified by acetone and ammonium sulphate fractionation, followed by ion-exchange to replace the excess of sulphate ions with acetate ions. Since then, the Swiss workers have used this method to prepare crystalline α -amylases from a variety of sources e.g. human saliva and pancreas [Meyer et al. (1948), Fischer et al. (1950)], Aspergillus oryzae [Fischer and de Montmollin (1951)] and Bacillus subtilis [Fellig et al. (1957)]. In the purification of the fungal and bacterial amylases, it was found necessary to include a "mixed-salt" fractionation using sodium chloride and ammonium sulphate, to separate active α -amylase from an inert, denatured form of the enzyme.

"Mixed-salt" precipitations (sodium sulphate-ammonium sulphate), followed by acetone and ammonium sulphate

fractionations, have been used to prepare α -amylases from other bacteria e.g. from B. coagulans [Campbell (1954)] and B. stearothermophilus [Manning and Campbell (1961)], while fractionation with ethanol has been employed in the purification of pancreatic amylase [Caldwell et al. (1952)] and B. subtilis amylase [Babbar et al. (1962)].

Methods involving the adsorption of α -amylase on starch have also been of importance in the purification of such enzymes [Markovitz et al. (1956), Dube and Nordin (1961)]. Indeed, adsorption was used in the preparation of the only plant α -amylase so far crystallised i.e. malted barley α -amylase [Schwimmer and Balls (1949)].

The main problem in the preparation of plant α -amylases is the removal of contaminating β -amylase. Dube and Nordin (1961) claim to have separated the two activities by adsorption of the enzymes on a column of defatted potato starch, and subsequent elution of the α -amylase with calcium acetate solution. However, a more widely-used method involves heating the enzyme mixture at 70°C in the presence of calcium ions [Ohlsson (1926), Myrbäck (1948)]. The calcium stabilises the α -amylase, while rendering the β -amylase more susceptible to heat denaturation [Kneen et al. (1943)]. α -Amylases from germinated barley [Schwimmer and Balls (1949)], rye [Ohlsson and Uddenberg (1933)] and wheat [Stamberg and Bailey (1938), Walden (1954)]

have been prepared free from β -amylase activity in this way.

A newer method of α -amylase purification described by Loyter and Schramm (1962) involves the specific precipitation of α -amylases from impure preparations by the formation, in ethanol, of an insoluble glycogen-amylase complex. It was shown that complex-formation yielded highly-purified enzymes, with specific activities as high as those of the best crystalline preparations.

This procedure has been adapted by MacGregor (1964) for use, along with acetone fractionation and heat-treatment at 70°C in the presence of calcium, in the preparation of α -amylases from plants, particularly those which contain little of the enzyme e.g. soya- and broad-beans. The purification scheme of MacGregor has also proved useful for the α -amylase from ungerminated and malted barley [Greenwood and MacGregor (1965)].

However, little is yet known of the action-patterns of the plant α -amylases. This Section describes the purification of the α -amylases from beans and ungerminated cereals, as a preliminary to a study of their properties and action-patterns. The enzymes were purified by the method of MacGregor, and, in order to make a direct comparison between the α -amylases of dormant and germinated cereals, the amylases of malted barley and wheat were also prepared by this procedure.

Experimental and Results

3a.

Soya- and Broad-Bean α -Amylases

These α -amylases were extracted and purified by the method of MacGregor (1964).

Dry, defatted bean flour (300 g.*) was shaken with aqueous calcium chloride solution (1200 ml.; 0.2%) and a little n-octanol (1 ml., to prevent foaming) for 4 hours at 18°C. After centrifugation at 900 g* for 30 minutes, the supernatant solution was cooled and fractionated with acetone at -5°C, fractions being removed on a centrifuge (-5°C; 1100 g) at 30, 40, 45, 50, 55, 60, 65 % acetone (V/V). The precipitates were air-dried to remove excess of acetone, and dissolved in distilled water.

These fractions of the soya-bean preparation containing most of the α -amylolytic activity (fractions at 50 and 55% acetone) were heated in 10 ml. buffered aliquots (pH 5.6; 0.02 M acetate buffer) containing calcium acetate (20 mg.) at 70°C for 20 minutes, quickly cooled to room temperature and centrifuged (1500 g). The heat-treatment completely deactivated the β -amylase contaminant of the soya-bean preparations, while the α -amylase activity was retained. This stage of the purification procedure was unnecessary in the broad-bean preparation, since there is no β -amylase in these beans.

* g. \equiv grams

g \equiv gravity

The heat-treated soya fractions and the most active broad-bean fractions (obtained at 50, 55 and 60% acetone) were cooled and refractionated with acetone at -5°C , at 35, 42, 47, 52, 57, 62 and 70% acetone (V/V).

The subfractions with the highest α -amylase activities (precipitated at 57 and 62% acetone and 52 and 57% acetone from the soya- and broad-bean solutions respectively) were further purified by the formation of glycogen-amylase complexes [Loyter and Schramm (1962)].

Ethanol was added slowly with stirring to the enzyme solution at 2°C , to a final concentration of 40% (V/V). After 15 minutes the mixture was centrifuged (2°C ; 1100 g), then phosphate buffer (1 ml.; 0.2 M; pH 8.0) and glycogen solution (0.2 ml.; 2%) were added to the supernatant liquid. The suspension was stirred for 10 minutes, the precipitated complex was removed at 1100 g, and dissolved in distilled water containing calcium acetate (0.2%). After incubation of this solution at 35°C for 6 hours to facilitate hydrolysis of the glycogen, the enzyme preparation was dialysed against calcium acetate solution (0.2%; 3 x 500 ml.) at 2°C for 3 days, and finally stored at 2°C .

The specific activities of the bean α -amylases after each stage of the purification process are shown in Table 3.01.

Table 3.01

Procedure	Original extract	First acetone fractionation	Heat treatment	Second acetone fractionation	Glycogen-complex formation
Soya-bean α -amylase Specific activity (units/mg./ml.)	0.7	20	36	110	700
Broad-bean α -amylase Specific activity (units/mg./ml.)	0.5	6.6	-	37	390

3b.

Oat α -Amylase(i) Initial attempt to prepare oat α -amylase

The method described above for the bean enzymes was used in an attempt to purify oat α -amylase.

An aqueous extract was prepared from dry, defatted oat flour (300 g.) and, after centrifugation, the solution was fractionated with acetone at -5°C . Specific activities of the fractions obtained were as shown in Table 3.02.

Table 3.02

Fraction number	0	1	2	3	4	5	6	7
Acetone concentration % (V/V)	0	30	35	40	45	50	55	63
α -amylase Specific activity (I.D.C. units/mg./ml. of digest)	2.0	15	22	22	23	14	2.9	1.8
β -amylase Specific activity (mg. maltose/mg./ml. of digest)	1.4	5.3	9.0	12.2	13.3	6.5	6.1	4.7

Aliquots (5 ml.) of fractions 1-4, buffered at pH 5.6, were heated with calcium acetate (20 mg.) for 20 minutes at 70°C, quickly cooled and centrifuged at 1500 g. When enzymic activities of the supernatant liquors had been measured, it was found that only 20% of the α -amylase activity had been recovered, although the β -amylase was completely deactivated.

The effect of varying the heating conditions was studied, in an attempt to improve the yield of active α -amylase. Addition of excess of calcium acetate (100 mg./5 ml. of solution) had no effect on the active yield, while buffering at pH 4.0 (0.02 M acetate buffer) or pH 6.8 (McIlvaine's buffer) decreased the activity to 8% of the original. In all cases the β -amylase was completely deactivated.

Heating for less than 20 minutes, or at temperatures below 70°C (e.g. 60 and 65°C) gave an improved recovery of α -amylase (50-60%), but active β -amylase could then be detected in the heated solutions.

When a portion of the original unfractionated extract was heated at 70°C for 20 minutes, 30% of the α -amylase was recovered, but again the β -amylase in the solution was incompletely deactivated. Here, excess of inert protein was protecting both amylases against heat denaturation.

Finally, it was found that, if the original extract were fractionated in the cold at 47% (V/V) acetone and a solution of this fraction given the same heat-treatment as the soya-bean fractions, then the β -amylase was deactivated, while a 38% recovery of the α -amylase activity was obtained. This was the method used in the final preparation of the oat α -amylase.

(ii) Final preparation of oat α -amylase.

An aqueous extract was prepared by shaking dry, defatted oat flour (400 g.) with calcium chloride solution (1200 ml.; 0.2%) and n-octanol (1 ml.) for 5 hours at 18°C, cooling the mixture to 5°C and centrifuging at 900 g. After the clear extract had been cooled to -5°C, 47% (V/V) acetone was added and the precipitate removed by centrifugation (-5°C; 1500 g). The precipitate, which was then air-dried, was dissolved in distilled water (235 ml.) to give a protein concentration of ca. 10 mg./ml.. This solution was next subjected to a heat-treatment similar to that used in the soya-bean α -amylase preparation.

Table 3.03

Procedure	Volume (ml.)	Activity (units/ml.)		Total units of activity		α -amylase Specific activity (units/ mg./ml.)	Yield (%)	
		α -amylase	β -amylase	α -amylase	β -amylase		α -amylase	β -amylase
Initial extract	685	4.0	0.77	2,740	530	2.2	100	100
47% acetone fraction	235	7.4	1.6	1,780	375	5.3	65	71
Heat treatment	245	2.8	0	690	0	3.2	25	0

The purification and recovery of activity after each stage of the procedure are shown in Table 3.03.

The heated extract was cooled and fractionated with chilled acetone. The α -amylase specific activities of these fractions are given in Table 3.04.

Table 3.04

Fraction number	1	2	3	4	5	6	7
Acetone concentration % (V/V)	25	30	35	40	45	50	67
α -amylase specific activity	0	8.9	7.1	9.3	6.0	1.0	0

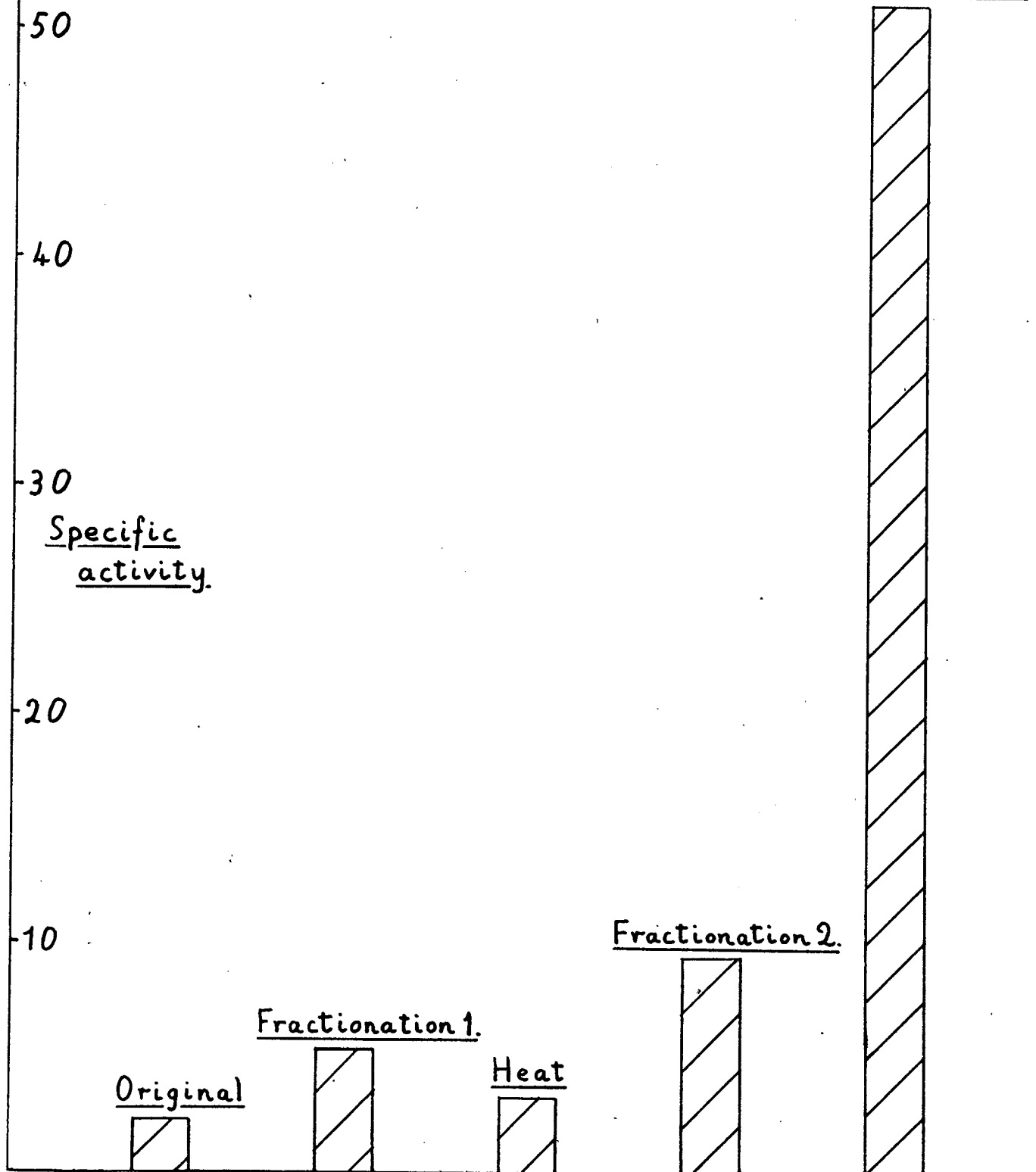
Fractions 4 and 5, which contained most of the amylolytic activity, were refractionated with cold acetone. The specific activity of each fraction was as in Table 3.05. Fractions were not taken at 37 and 42% acetone, since no protein appeared to precipitate at these concentrations of non-solvent.

Table 3.05

Fraction number	(4+5)1	(4+5)2	(4+5)3	(4+5)4	(4+5)5	(4+5)6
Acetone concentration % (V/V)	27	32	47	52	57	67
α -amylase specific activity	51	27	20	10	6	0

FIG. 3.1

Purification of oat α -amylase. Fractionation 3.



Attempts were made to purify the enzyme further by glycogen-complex formation in 40% ethanol. However, all such attempts proved unsuccessful, and experiments to form the complex in 32% acetone (the solubility limit of the glycogen) also failed.

The purification obtained at each stage of the procedure is shown in Fig. 3.1.

3c.

Rye α -Amylase

Rye α -amylase was extracted and purified by a method similar to that outlined above for the oat enzyme.

Dry, defatted rye flour (300 g.) was extracted for 5 hours with aqueous calcium chloride solution (1000 ml.; 0.2%), centrifuged and a 50% acetone fraction obtained. After the air-dried precipitate had been dissolved in distilled water (310 ml.) to bring the protein concentration to ca. 10 mg./ml., the solution was heated in the same manner as the soya-bean and oat enzymes.

The purification and yields of the enzyme products were as shown in Table 3.06.

Table 3.06

Procedure	Volume (ml.)	Activity (units/ml.)		Total units of activity		α -amylase Specific activity (units/ mg./ml.)	Yield (%)	
		α -amylase	β -amylase	α -amylase	β -amylase		α -amylase	β -amylase
Initial extract	680	40	20	27,200	13,600	15	100	100
50% acetone fraction	310	35	9.1	10,900	2,820	27	40	21
Heat treatment	320	16	0	5,100	0	16	19	0

The enzyme solution was cooled and fractionated at -5°C with acetone. The specific activities of the α -amylase in these fractions are given in Table 3.07.

Table 3.07

Fraction number	1	2	3	4	5	6
Acetone concentration % (V/V)	30	40	50	60	70	80
α -amylase specific activity	37	71	60	72	21	0

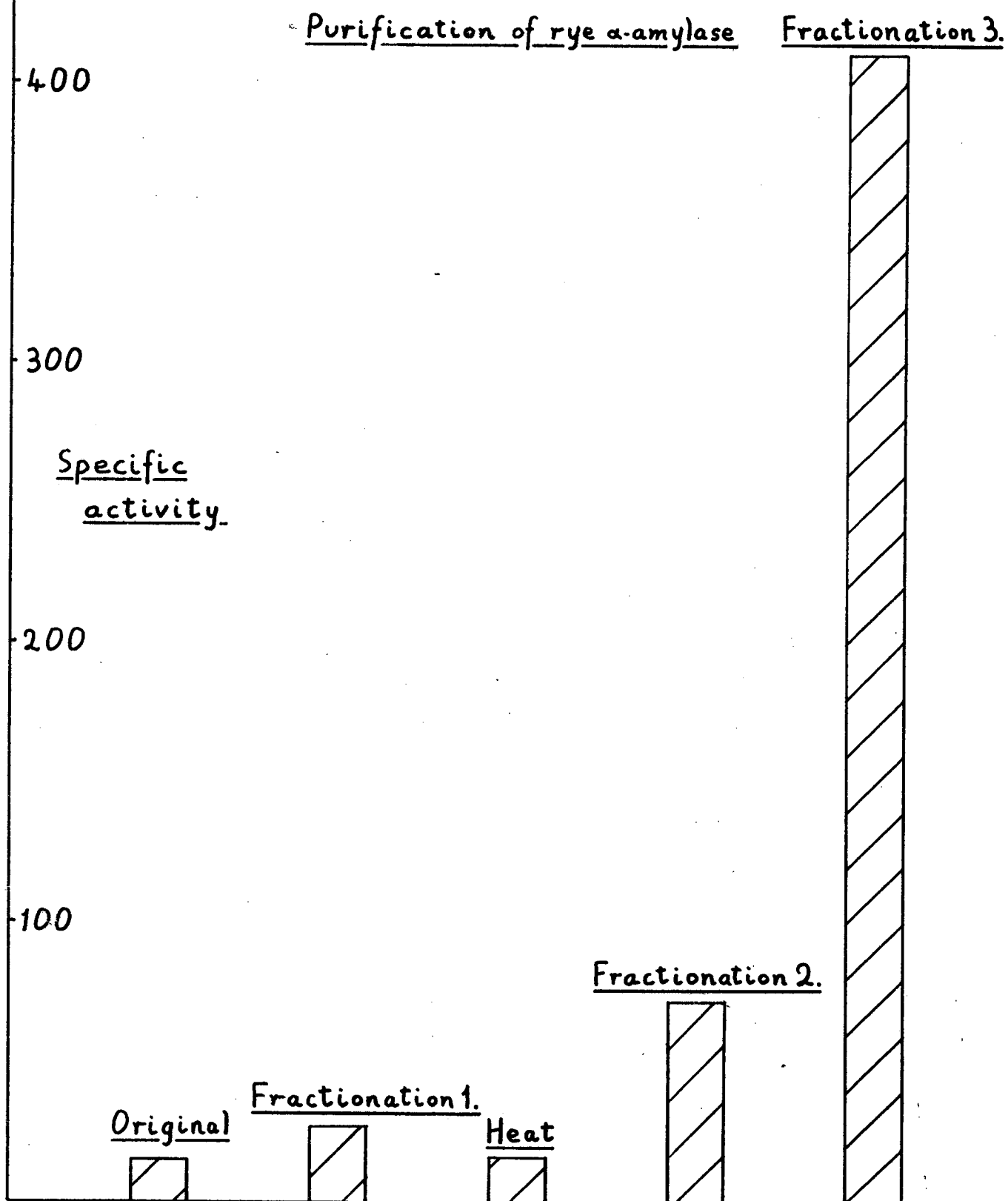
Fraction 4, containing most of the α -amylase activity, was refractionated with cold acetone, and Table 3.08 shows the activities of the fractions.

Table 3.08

Fraction number	4.1	4.2	4.3	4.4	4.5	4.6
Acetone concentration % (V/V)	25	35	45	55	65	75
α -amylase specific activity	150	280	410	130	120	40

As in the preparation of the oat enzyme, attempts to purify the α -amylase further by glycogen-complex formation proved unsuccessful.

FIG. 3.2



The purification obtained at each stage is shown in Fig. 3.2.

3d.

Wheat α -Amylase

An extract was prepared from defatted wheat flour (300 g.) in a manner similar to that described in the preparation of α -amylase from rye; this extract was fractionated at 50% acetone and the resulting precipitate was dissolved in distilled water to give a protein concentration of ca. 10 mg./ml.. The solution was then heat-treated at 70°C.

The yields and purification of the enzymes obtained after each stage are shown in Table 3.09.

Table 3.09

Procedure	Volume (ml.)	Activity (units/ml.)		Total units of activity		α -amylase Specific activity (units/ mg./ml.)	Yield (%)	
		α -amylase	β -amylase	α -amylase	β -amylase		α -amylase	β -amylase
Initial extract	834	17	58	14,200	48,400	6.9	100	100
50% acetone fraction	310	24	50	7,440	15,500	18	52	32
Heat treatment	333	9.2	0	3,040	0	11	21	0

The enzyme solution was then fractionated with chilled acetone and the α -amylolytic specific activities of the fractions are given in Table 3.10.

Table 3.10

Fraction number	1	2	3	4	5	6
Acetone concentration % (V/V)	30	35	40	45	50	60
α -amylase specific activity	0	17	20	44	56	6

Fractions 4 and 5 were refractionated with acetone and the resultant purification was as below:

Table 3.11

Fraction number	(4+5)1	(4+5)2	(4+5)3	(4+5)4	(4+5)5	(4+5)6
Acetone concentration % (V/V)	32	37	42	47	52	60
α -amylase specific activity	180	240	220	260	80	6

As was found for the oat and rye enzymes, no glycogen-complex could be formed.

The purification obtained at each stage is shown in Fig. 3.3.

FIG. 3.3

Purification of wheat α -amylase

Fractionation 3.

250

200

150

Specific
activity.

100

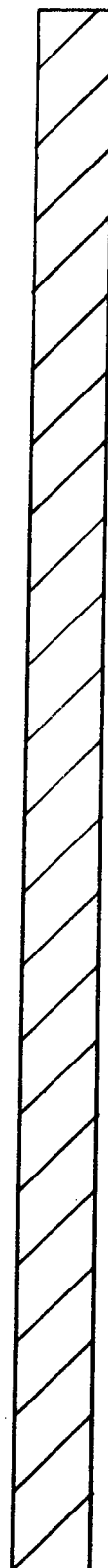
50

Original

Fractionation 1.

Heat

Fractionation 2.



3e. Malted Barley and Wheat α -Amylases

(i) Preparation of barley and wheat malts

Samples (100 g.) of barley and wheat were left at 20°C for 3 days in tap water (300 ml.). Each day the water was decanted and then replaced. After three days, the water was removed, and the grain was maintained at 20°C for a further 5 days, when much sprouting took place. The germinated cereals were kilned in an oven for 24 hours at 50°C, and then for 24 hours at 60°C. The dry seed was rubbed and sieved to remove the shoots and rootlets, then finely ground into flour which was defatted with solvent ether.

(ii) Preparation of the malt α -amylases

Extracts were prepared as usual from dry, defatted flour (100 g.) and aqueous calcium chloride solution (330 ml.; 0.2%). These extracts were heated at 70°C in 10 ml. portions, *Added pH 5.6 buffer* buffered at pH 5.6, with calcium acetate (20 mg.) for 35 minutes (barley extract) or 25 minutes (wheat extract). The yields and purification of the enzymes after these stages are shown in Tables 3.12 and 3.13.

Table 3.12

Malted barley extract

Procedure	Volume (ml.)	Activity (units/ml.)		Total units of activity		α -amylase Specific activity (units/mg./ml.)	Yield (%)	
		α -amylase	β -amylase	α -amylase	β -amylase		α -amylase	β -amylase
Initial extract	225	1,400	4.4	315,000	990	550	100	100
Heat treatment	235	1,200	0	282,000	0	710	90	0

Table 3.13

Malted wheat extract

Procedure	Volume (ml.)	Activity (units/ml.)		Total units of activity		α -amylase Specific activity (units/ mg./ml.)	Yield (%)	
		α -amylase	β -amylase	α -amylase	β -amylase		α -amylase	β -amylase
Initial extract	250	5,200	38	1,300,000	9,500	1,300	100	100
Heat treatment	260	1,600	0	416,000	0	500	32	0

The heated solutions were fractionated with cold acetone, and the α -amylolytic activities of the fractions are shown in Table 3.14.

Table 3.14

Fraction number	1	2	3	4	5	6	7
Acetone concentration % (V/V)	30	35	40	45	50	60	70
Malted barley α -amylase specific activity	900	1,500	3,100	8,500	16,000	2,300	120
Malted wheat α -amylase specific activity	820	2,400	3,800	7,300	7,400	1,000	45

Fractions 4 and 5 were combined and refractionated with chilled acetone. The purification obtained is given below.

Table 3.15

Fraction number	(4+5)1	(4+5)2	(4+5)3	(4+5)4	(4+5)5	(4+5)6	(4+5)7
Acetone concentration % (V/V)	32	37	42	47	52	57	65
Malted barley α -amylase specific activity	19,000	22,000	37,000	80,000	73,000	30,000	6,000
Malted wheat α -amylase specific activity	21,000	33,000	42,000	74,000	58,000	23,000	5,400

Subfractions (4+5)4 were further purified by the formation of glycogen-amylase complexes. Phosphate buffer (0.5 ml.; 0.2 M; pH 8.0) and ethanol, to a final concentration of 40% (V/V), were added to the enzyme solution (10 ml.) at 2°C, the mixture was stirred for 15 minutes and centrifuged (2°C; 1100 g). To the supernatant liquid was added glycogen solution (0.2 ml.; 2%), the suspension was stirred for 5 minutes and the precipitated complex was removed at 1100 g. This complex was dissolved in aqueous calcium acetate solution (0.2%) and the enzyme preparation was left at 18°C for 4 hours to facilitate hydrolysis of the dissolved glycogen. Finally the mixture was dialysed against calcium acetate solution (0.2%; 2 x 800 ml.) at 2°C for 3 days, then stored at 2°C.

The specific activities of the malted barley and wheat α -amylase preparations after glycogen-complex formation were 130,000 and 140,000 respectively.

The purification obtained at each stage of the procedure is shown in Figs. 3.4 and 3.5.

3f. α -Amylases from hog pancreas and *Bacillus subtilis*

Crystalline α -amylases from hog pancreas and *Bacillus subtilis* were obtained through Sigma London Chemical Co. Ltd., Lettice Street, London.

The specific activities of these enzymes at 35°C and pH 5.5 were 44,000 and 180,000 respectively (in I.D.C. units/mg./ml.).

FIG. 3.4

Purification of malted barley α -amylase

Glycogen
complex

125

100

75

Specific
activity ($\times 10^{-3}$)

50

25

Fractionation 2.

Fractionation 1.

Original ($\times 10$)

Heat ($\times 10$)

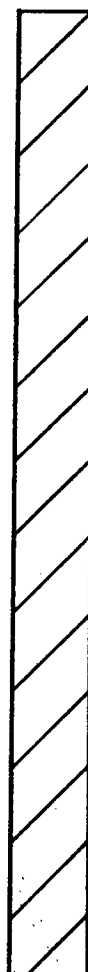
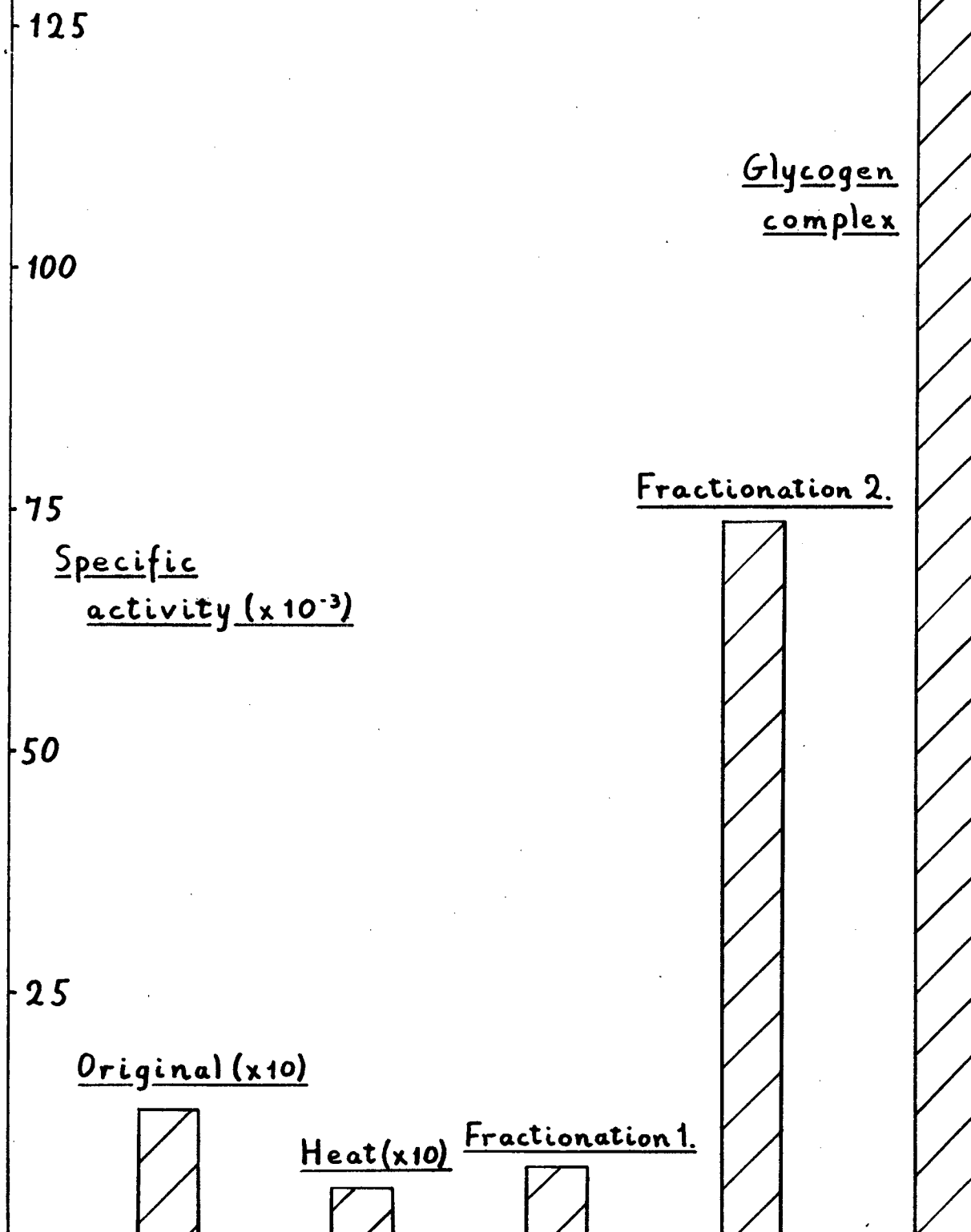


FIG. 3.5

Purification of malted wheat α -amylase



3g.

Purity of the cereal α -amylases

The enzyme solution of highest specific activity from each source was used for the experiments described in this Section and Sections 4 and 5.

Digests were set up as follows:

(i) Test for β -amylase activity: Acetate buffer (2 ml.; 0.2 M; pH 3.6) and enzyme solution (0.5 ml. containing 2-8 units ungerminated cereal enzyme or 0.025 ml. containing 45 units malted cereal enzyme) were incubated at 35°C for 45 minutes, then freshly-prepared soluble starch solution (15 ml.; 0.6%; at 35°C) was added. 1 ml. samples were withdrawn immediately after addition of the starch and after 4 hours, and the reducing power was determined by the alkaline ferricyanide method (See Section 2d.).

No increase in reducing power of the digests was found in 4 hours, indicating the absence of β -amylase in the α -amylase preparations.

(ii) Test for maltase activity: Maltose (2 mg.) and enzyme solution (1 ml.; 1 unit ungerminated cereal α -amylase or 45 units malted cereal enzyme) were incubated at 35°C under toluene. At intervals aliquots (0.025 ml.) were removed for chromatographic investigation (See Section 2f.).

After 90 hours' incubation of the digests containing oat, rye or wheat enzyme, or after 20 hours' incubation of the digests with malted cereal α -amylase, maltose was the only sugar which could be detected on the chromatograms. It may therefore be concluded that maltase is absent from the α -amylase solutions.

(iii) Test for D-enzyme activity: Maltotetrose (1 mg.) and enzyme solution (0.5 ml.; 2 units ungerminated cereal enzyme or 45 units malted cereal enzyme) were incubated at 35°C under toluene. 0.125 ml. samples were removed after 45 and 96 hours for examination by paper chromatography (See Section 2f.).

In each case, traces of glucose, maltose and maltotriose were present, but no oligosaccharide larger than the tetrose was found in the digest. This showed that there was no detectable amount of D-enzyme contaminating the α -amylases.

(iv) Test for phosphorylase activity: Acetate buffer (1 ml.; 0.2 M; pH 5.5) and disodium hydrogen phosphate solution (1 ml.; 0.26%), amylose solution (5 ml.; 0.1%) and enzyme solution (0.1 ml. containing 1 unit or 45 units) were incubated at 35°C under toluene. The free phosphate concentration of 2 ml. samples was tested after 20 hours (malted cereal enzyme digests) or 72 hours (ungerminated cereal enzyme digests) by the method of Allen (1940).

The 2 ml. samples were pipetted into 25 ml. standard flasks to which was added perchloric acid (2 ml.; 60%), amidol solution (2 ml.; 1% amidol in 20% sodium bisulphite solution) and ammonium molybdate solution (1 ml.; 8.3%). The solution volumes were made up to 25 ml. with distilled water, and after 10 minutes the absorption values were measured using an "EEL" colorimeter (Red filter No. 608).

The absorption values of the enzyme digests were found to be the same as those of control digests containing no

enzyme. It can thus be inferred that there is no phosphorylase in the α -amylase preparations.

Discussion

Soya- and broad-bean α -amylases: The method of MacGregor (1964) was used successfully to prepare the bean α -amylases; the specific activities of the soya- and broad-bean enzymes were increased by factors of ca. 1,000 and 780, respectively (Table 3.01). The final specific activities of the enzymes were very similar to those obtained by MacGregor.

Oat α -amylase: An initial attempt to purify this enzyme by the procedure described for the beans was unsatisfactory (p. 22). Heating of the acetone-fractions, whilst deactivating the β -amylase, caused an 80% loss of the α -amylase activity. The oat enzyme is, therefore, more heat-labile than that of soya-beans at the same stage of purification; the recovery of soya α -amylase activity after heating was ca. 90%. The amount of active oat α -amylase obtained could not be improved by the addition of an excess of calcium ions before heating, nor by changing the pH of the solution. When the time, or temperature, of heating was decreased, a greater quantity of active α -amylase was recovered, but the β -amylase was then not completely denatured.

Greenwood and MacGregor (1965) showed that the presence of inert protein from germinated barley protects the

α -amylase against heat denaturation, and so a portion of the original oat extract, containing much more inert protein than the acetone-fractions, was heated. The yield of α -amylase was improved, but active β -amylase could still be detected in the heated solution. The inert protein of the crude extract protected both the α - and β -amylases against deactivation.

It was found more satisfactory to fractionate the oat extract at 47% acetone, then to heat a solution of this fraction (protein concentration ca. 10 mg./ml.) at 70°C. Sufficient inert protein was present to give protection to the α -amylase, while there was not enough to prevent denaturation of the β -amylase.

This procedure was adopted in the final preparation of the α -amylases of oats, rye and ungerminated wheat. Care was taken to ensure that the concentration of protein in the solutions to be heated was the same in each case (ca. 10 mg./ml.).

The α -amylolytic specific activity of the oat preparation decreased at the heat-treatment stage, as relatively more α -amylase activity was lost from the solution than inert protein. It was essential, however, to include this procedure in the purification scheme, as it is the only satisfactory method of removing β -amylase activity.

Although most of the α -amylase activity was precipitated between acetone concentrations of 30 and 40% (Table 3.04),

on subfractionation the enzyme was precipitated at 27% acetone (Table 3.05). In general, an enzyme will precipitate at the same acetone concentration from two solutions, only if the ion and protein concentrations of these solutions are the same. The solubility of α -amylases in acetone is increased by the presence of calcium ions, and thus the α -amylase in a subfraction would be expected to precipitate at a lower acetone concentration than that in a solution containing 0.2% calcium acetate (i.e. the heated enzyme solution).

Attempts to purify the enzyme further by glycogen-complex formation in ethanol or acetone were unsuccessful. In 1964, Levitzki, Heller and Schramm reported that the glycogen-complex does not precipitate if an excess of substrate is present. It is likely, then, that the failure to obtain a complex in this case was due to the small amounts of enzyme (ca. 40 units) present in the solutions tested.

The procedure gave an overall purification factor of 23, and yielded a product with specific activity comparable to that of the α -amylase prepared from barley by Greenwood and MacGregor (1965). It is interesting to note that, in this case also, no glycogen-complex could be formed.

Rye α -amylase: The method used for the rye α -amylase was very similar to that for the oat enzyme.

The initial extract contained ca. 10 times more α -amylase than the oat extract (Compare Tables 3.03 and 3.06). Proportionally less α -amylase was obtained in the first acetone-fraction than in the oat preparation, but more rye enzyme was retained on heating, so that the percentage yields after the first two stages of purification were comparable.

Again, the specific activity of the preparation decreased on heating, as the loss of α -amylolytic activity outweighed the loss of inert protein.

The enzyme of highest specific activity precipitated at higher acetone concentrations than did the oat α -amylase (Compare Tables 3.07, 3.08 and 3.04, 3.05), but this is more likely to be caused by differences in the nature of the contaminating protein, than by differences in the two α -amylases. As in the preparation of the oat enzyme, the rye α -amylase precipitated from the subfraction solution at lower acetone concentration than from the newly-heated solution containing calcium acetate (0.2%).

As for the oat α -amylase, no glycogen-complex precipitated from an ethanolic solution. In the experiments with the rye enzyme, greater amounts of amylase (100 units) were available for complex-formation; indeed the total number of units of rye α -amylase in the solutions was comparable to that in broad-bean preparations, where a complex did precipitate. It must therefore be concluded that the source of the enzyme influences the formation of

a glycogen-complex more than the amount (in terms of units of activity) of amylase present.

The rye enzyme was purified by a factor of 27, and the purest product obtained had a higher specific activity (410 I.D.C. units/mg./ml.) than that from the oat extract (51 I.D.C. units/mg./ml.).

Wheat α -amylase: The crude extract contained less α -amylase, but more β -amylase, than the extract of rye flour (Compare Tables 3.06 and 3.09). After two stages of purification, the percentage yield of active α -amylase was very similar to those obtained in the oat and rye preparations, although the wheat enzyme was less heat-stable than that of rye.

The specific activity decreased on heating, but for each cereal amylase the specific activity of the enzyme in the heated solution was greater than that in the original extract.

During the acetone fractionations, the wheat α -amylase of highest specific activity precipitated at approximately the same acetone concentrations as did the rye enzyme (Compare Tables 3.07, 3.08 and 3.10, 3.11). The amylase in the subfraction containing little calcium was insoluble at a lower concentration of acetone than that in the heated solution.

No glycogen-complex was obtained from solutions containing 100 units of enzyme.

The specific activity of the wheat α -amylase was increased by this procedure by a factor of 38, and the highest specific activity obtained (260 units/mg./ml.) was intermediate between those of the oat and rye enzymes (51 and 410 units/mg./ml., respectively).

Malted barley and malted wheat α -amylases: The increase in the active α -amylase content of cereals such as barley and wheat on germination has been known for many years [Mayer and Klinga-Mayer (1940), Kneen (1944)].

Here, it was found that an aqueous extract from 100 g. of malted wheat flour contained ca. 100 times more active α -amylase than an extract from 300 g. of flour from the original wheat (Compare Tables 3.09 and 3.13). There was, however, no corresponding increase in the amount of β -amylase present.

The malted barley flour yielded less amylase than the corresponding wheat preparation (Tables 3.12 and 3.13), but much more than flour from the ungerminated cereals (Compare Tables 3.03, 3.06, 3.09 and 3.12).

The malt extracts were not fractionated with acetone before heating, because it was likely the α -amylolytic specific activity would be so high after fractionation that the enzyme would be extremely heat-labile. Indeed, MacGregor (1964) fractionated a malted barley extract with acetone and found, on heating, that 60% of the α -amylase activity was lost. It can be seen in Table 3.12 that, by heating the original extract, a recovery of 90% of the

α -amylolytic activity was obtained. A similar high yield (97%) was obtained by Schwimmer and Balls (1949) after heat-treatment of a crude malt syrup at 70°C for 15 minutes. However, the malted wheat α -amylase was less heat-stable, and only 30% of the activity was recovered. These findings are in agreement with results of Kneen et al. (1943), who reported that the α -amylase of malted barley has a greater heat-stability than that of malted wheat.

It must be stressed that the stability of cereal α -amylases is very dependent on the presence of calcium, and the nature and concentration of other proteins present in the enzyme solution [Greenwood and MacGregor (1965)]. Thus the difference in the heat-stabilities of the α -amylases of malted barley and wheat cannot be regarded as evidence that the enzymes are not identical. Considering the similarities between the α -amylases (Sections 4 and 5), it must rather be concluded that the difference lies in the nature of the contaminating proteins. The crude barley extract may contain a stabilising factor, capable of protecting the α -amylase against heat denaturation or, in the extract from the wheat, there may be more proteases which can degrade the amylase.

The malt extracts were heated for longer than the enzyme solutions from ungerminated cereals (35 and 25 minutes, instead of 20 minutes) in an attempt to ensure complete deactivation of β -amylase.

As little malted barley α -amylolytic activity was lost on heating and inert protein was removed from the solution,

the specific activity of this preparation increased (Table 3.12). In the case of the malted wheat enzyme, the specific activity decreased because of the high loss of α -amylase activity (Table 3.13).

In the acetone fractionations, the purest malted cereal α -amylases precipitated at approximately the same concentrations of acetone as did the wheat enzyme (Compare Tables 3.10, 3.11 and 3.14, 3.15).

Glycogen-complexes were obtained and gave enzymes of high specific activity. It must be noted that the solutions from which these complexes precipitated contained $20-40 \times 10^3$ units of enzyme.

In 1964, Levitzki et al. showed that the enzyme-glycogen complex is soluble if an excess of the polysaccharide is present, and the range of the enzyme:glycogen ratio suitable for precipitation is narrow i.e. if a 10-fold change from the optimum value of the ratio is made, little or no complex will precipitate. As complexes were obtained from the malted cereal α -amylase preparations, it may be concluded that the ratio enzyme ($20-40 \times 10^3$ units):glycogen (4 mg.) is favourable for precipitation, while a ratio of less than enzyme (20×10^2 units):glycogen (4 mg.) must be unfavourable.

In the attempts to form complexes with the ungerminated cereal amylases, the ratio was enzyme (40-100 units):glycogen (4 mg.). Then on the basis of this enzyme:glycogen ratio, no complex would be expected to precipitate.

Also, since the bean enzymes did give complexes when the enzyme:substrate ratio was of the order enzyme (100 units):glycogen (4 mg.), it may be concluded that these α -amylases are not the same as those from cereals.

The overall purification factors of the malted barley and wheat α -amylases were ca. 240 and 110, respectively. The specific activities of the final products compare favourably with that of a commercial, crystallised α -amylase from B. subtilis (p. 36).

The specific activities of the α -amylases from oats, rye and wheat are lower than those of the malted cereal enzymes, but the results in Sections 4 and 5 show that all the cereal amylases are very similar; thus the enzyme preparations from the ungerminated cereals must still contain much inert protein.

After dialysis, samples of the malted cereal enzymes were tested chromatographically for the presence of reducing sugars, but no such sugar was found.

Purity of the α -amylases (p. 37): The absence of contaminating carbohydrases in all the α -amylase preparations was shown by the following observations:

(i) Since the reducing power of a digest containing enzyme and starch at pH 3.6 did not increase, it may be concluded that the α -amylase did not contain a detectable amount of β -amylase.

(ii) The failure of an enzyme sample, when incubated with maltose, to produce glucose means that there was no maltase present.

(iii) In a digest, containing enzyme and maltotetrose, no oligosaccharide larger than the tetrose could be detected, indicating the absence of D-enzyme. This enzyme attacks maltotetrose to produce a mixture of sugars including glucose, maltotriose, maltopentose and higher maltodextrins [Whelan (1958)].

(iv) Phosphorylase, in the presence of phosphate ions, degrades amylose to produce glucose-1-phosphate, at the same time reducing the free phosphate content of the system [Barker and Bourne (1953)]. It may therefore be inferred that there was no phosphorylase present in the samples of α -amylase tested, since no decrease of the concentration of free phosphate was found in digests containing α -amylase, amylose and inorganic phosphate.

Section 4

General Properties of the α -Amylases

4.

Introduction

It is thought that α -amylases are metallo-enzymes, containing calcium as part of their molecular structure [Vallée et al. (1959), Stewart (1963)]. This calcium does not participate directly in the formation of the enzyme-substrate complex, but rather holds the enzyme molecule in the correct configuration for activity and maximum stability [Hsiu et al. (1964)]; the stabilising effect of calcium ions on α -amylases has been known for many years [Kneen et al. (1943), Caldwell and Kung (1953)].

Removal of the metal ion by chelating agents or dialysis leads to reversible inactivation, for the activity may be completely restored on the addition of calcium [Stein et al. (1964)], provided there are no proteolytic enzymes present. Calcium-deficient α -amylases are susceptible to attack by proteases, while the native enzymes are remarkably resistant [Stein and Fischer (1958)].

The strength of binding of calcium ions to the protein varies according to the source of the amylase, decreasing in the order fungal, bacterial, mammalian, malted cereal [Fischer and Stein (1960)]. Thus, the first three types of enzyme normally contain sufficient calcium, so that the addition of an excess produces no activation, while the metal-enzyme binding of malt α -amylase is so weak that added calcium often has an activating effect [Meyer (1952)]. For all α -amylases, however, the presence of an excess of calcium increases the stability of the

enzymes towards acid, heat, or urea denaturation [Fischer and Stein (1960)].

In general, α -amylases are quickly and irreversibly denatured by acid, but are stable at pH values between 5 and 8.5 [Meyer (1952)]; exceptions are the enzymes of Aspergillus oryzae and swine pancreas, which are unstable at pH values less than 5.5 and 6 respectively [Fischer and de Montmollin (1951 a), Fischer and Bernfeld (1948)].

The mammalian amylases exhibit maximum activity at pH 6.9 in the presence of chloride ions [Fischer and Bernfeld (1948), Bernfeld et al. (1948 and 1950)], but the value of this pH-optimum can be changed by the presence or absence of certain anions [Myrbäck (1926)]. The α -amylases of Bacillus subtilis and A. oryzae have activity-optima in the ranges pH 5.2-6.4 and 5.5-5.9 respectively [Menzi et al. (1957), Fischer and de Montmollin (1951 a)], while for the enzyme from malted barley this range is pH 4.7-5.4 [Fischer and Haselbach (1951)], or 5.5 [Greenwood and MacGregor (1965)].

As the temperature of an amylase digest is increased, two opposing effects may be observed: the amylolytic activity increases, but so also does the rate of heat-denaturation of the enzyme. Above 50°C, the second effect predominates (except for the α -amylase of B. stearothermophilus, which is very heat-stable), and the enzyme activity appears to decrease with increasing temperature. Thus the α -amylases exhibit maximum activity at temperatures

between 40 and 55°C [Bernfeld et al. (1948), Fischer and Haselbach (1951), Menzi et al. (1957), MacGregor (1964)], although the exact value of the optimum temperature depends on the time taken to test the enzymic activity, as well as on the concentration of calcium and inert protein in the digest. Activation energies of 7-14 Kilocalories/mole have been reported for α -amylases [Whelan (1958), MacGregor (1964)].

In 1921, Olsson showed that malt α -amylase is inhibited by silver nitrate, marked inhibition being observed even with concentrations of silver as low as $2 \times 10^{-7}M$. Later workers have described the inhibition of amylases by heavy metals such as silver, mercury, copper and lead [e.g. Di Carlo and Redfern (1947), Muus et al. (1956), Urata (1957), Stewart (1963)].

There is conflicting information concerning the nature of the amino acids responsible for the catalytic activity of α -amylases. However, it seems probable that carboxyl and imidazolium groups, i.e. aspartic or glutamic acids and histidine, are involved in the breakdown of the enzyme-substrate complex [Ono et al. (1958), Dube (1961), Thoma et al. (1963)]. Little and Caldwell (1942) presented evidence that free amino groups are necessary for the formation of the amylase-polysaccharide complex, and this has been confirmed by Di Carlo and Redfern (1947), Benner and Myrbäck (1952), Radichevich et al. (1959). Sulphydryl groups are not considered to be essential for



α -amylolytic activity [Caldwell et al. (1945)], although some workers have shown that reagents, thought to be specific for sulphydryl groups, are capable of inhibiting α -amylases [Di Carlo and Redfern (1947), Fischer and Haselbach (1951), Muus et al. (1956)].

The molecular weights of several α -amylases have been determined, and are on average ca. 50,000 e.g. 51,000 for the enzyme of A. oryzae; 45,000 for hog pancreatic amylase; and 49,000 for the monomeric form of B. subtilis amylase [Fischer and Stein (1960)]. However, Manning et al. (1961) obtained the value of 15,400 for the molecular weight of the α -amylase from B. stearothermophilus, while Schwimmer and Balls (1949) reported that the molecular weight of malted barley α -amylase is 59,500.

This Section describes a study of the general properties of the α -amylases from oats, wheat and rye. The nature of the active centres of these enzymes and also of the soya- and broad-bean amylases has been investigated. In addition, a comparison has been made of the molecular sizes of α -amylases from germinated and dormant barley and wheat.

Experimental and Results

4a. Effect of various reagents on α -amylolytic activity

(1) Effect of salts

Digests were prepared containing acetate buffer (1 ml.; 0.2 M; pH 5.5), enzyme solution (1-3 units) and the requisite amount of salt in water, in a total volume of 7 ml.. After incubation at 35°C for 2 hours, aliquots of warm amylopectin β -limit dextrin solution (1 ml.; 0.6%) were added to the digests, and the enzymic activities were determined in the usual manner. The results are shown in Tables 4.01 and 4.02.

Table 4.01

Effect of various salts

Salt	Concentration	Percentage inhibition		
		Oat α -amylase	Rye α -amylase	Wheat α -amylase
Sodium chloride	10^{-3} M	0	0	0
Calcium chloride	10^{-3} M	0	0	0
Potassium cyanide	10^{-3} M	0	0	0
Potassium nitrate	10^{-3} M	0	0	0
Potassium sulphate	10^{-3} M	0	0	0
Ammonium molybdate	10^{-3} M	70	90	90
	10^{-4} M	0	0	0

Table 4.02

Effect of salts of heavy metals

Salt	Concentration	Percentage inhibition		
		Oat α -amylase	Rye α -amylase	Wheat α -amylase
Copper sulphate	$10^{-4}M$	100	90	100
	$10^{-5}M$	0	10	30
	$10^{-6}M$	0	0	0
Lead acetate	$10^{-4}M$	20	0	0
	$10^{-5}M$	0	0	0
	$10^{-6}M$	0	0	0
Silver nitrate	$10^{-4}M$	100	100	100
	$10^{-5}M$	100	100	100
	$10^{-6}M$	25	100	100
	$10^{-7}M$	n.d.	100	40
	$10^{-8}M$	n.d.	30	0
Mercuric chloride	$10^{-4}M$	100	100	100
	$10^{-5}M$	100	100	100
	$10^{-6}M$	40	100	100
	$10^{-7}M$	n.d.	80	100
	$10^{-8}M$	n.d.	0	10

n.d. = not determined.

(11) Effect of McIlvaine's phosphate-citrate buffer

For the broad-bean and oat enzymes, digests contained buffer (1 ml.; McIlvaine's buffer; pH 5.5), amylopectin β -limit dextrin solution (1 ml.; 0.6%) and water in a total volume of 7.7 ml.. When the mixture had been incubated at 35°C for 30 minutes, 0.3 ml. enzyme solution (1 unit) was added to each digest and the activities were determined after 100 minutes. The amylolytic activities in control digests containing acetate buffer (0.2 M; pH 5.5) were also measured. In both cases, it was found that the α -amylase in McIlvaine's buffer retained only 35% of the activity of the control.

Experiments, similar to that above, carried out with broad-bean α -amylase in disodium hydrogen phosphate-acetic acid and sodium acetate-citric acid buffers, showed that phosphate-acetate buffer caused no loss of enzymic activity, while acetate-citrate buffer decreased the activity by 10%.

Further, it was found that if the broad-bean α -amylase were preincubated for 75 minutes at 18°C in McIlvaine's buffer (2 ml.; pH 5.5) prior to an activity determination at 35°C, then complete loss of activity was obtained.

For the rye and wheat enzymes, digests were prepared as follows: Buffer (1 ml.; McIlvaine's or acetate; pH 5.5), enzyme solution (2 units) and water in a total of 7 ml.. The mixtures were allowed to stand for 90 minutes at 18°C, then for 30 minutes at 35°C. Amylopectin β -limit dextrin

solution (1 ml.; 0.6%; at 35°C) was added to each digest and the enzymic activities were measured after 50 minutes.

It must be noted that the wheat α -amylase solution contained calcium acetate (2 mg./ml.).

The rye enzyme in McIlvaine's buffer retained 50% of the activity of the control, while the wheat enzyme retained 80% of the activity.

(iii) Effect of other reagents

Acetate buffer (1 ml.; 0.2 M; pH 5.5), enzyme solution (1-4 units), the reagent and water in a total of 7 ml. were incubated for 2 hours at 35°C; warm amylopectin β -limit dextrin solution (1 ml.; 0.6%) was then added and the amylolytic activities were determined.

The results are given in Table 4.03.

Table 4.03

Reagent	Concentration	Percentage inhibition		
		Oat α -amylase	Rye α -amylase	Wheat α -amylase
Ascorbic acid	$10^{-3}M$	100	100	100
	$10^{-4}M$	25	45	25
	$10^{-5}M$	0	0	0
	$10^{-6}M$	0	0	0
2-amino- 2 (hydroxymethyl)- propane-1:3-diol (tris buffer)	$10^{-3}M$	n.d.	0	0

n.d. \equiv not determined.

(iv) Effect of ethylene diamine tetraacetate (EDTA) and trypsin

Digests were prepared containing, in addition to acetate buffer (1 ml.; 0.2 M; pH 6.9), in a total volume of 7 ml.:

a EDTA solution (1 ml.; 10^{-1} M), trypsin (2 mg.; crystalline, B.D.H.) and α -amylase solution (1-3 units).

c EDTA solution (1 ml.; 10^{-1} M) and α -amylase solution (1-3 units).

e Trypsin (2 mg.; crystalline, B.D.H.) and α -amylase solution (1-3 units).

g Control: α -amylase solution (1-3 units).

Further digests b, d, f and h were prepared as a, c, e and g in a total volume of 6 ml.:

The mixtures were allowed to stand at 18°C for 2 hours, then aqueous calcium chloride solution (1 ml.; M) was added to digests b, d, f and h. All digests were set at 35°C for 30 minutes, then warm amylopectin β -limit dextrin solution (1 ml.; 0.6%) was added to each and the enzymic activities were determined. The results were as shown in Table 4.04.

Table 4.04

Digest	Reagents	Percentage inhibition		
		Oat α -amylase	Rye α -amylase	Wheat α -amylase
a	EDTA+trypsin	100	100	100
b	EDTA+trypsin+calcium	95	0	60
c	EDTA	100	100	100
d	EDTA+calcium	85	0	50
e	Trypsin	45	40	45
f	Trypsin+calcium	75	50	50

For the rye α -amylase, further digests were prepared by adding to acetate buffer (1 ml.; 0.2 M; pH 6.9) and distilled water (10 ml.):

i and j EDTA solution (0.1 ml.; 10^{-1} M), trypsin (1 mg.; crystalline, B.D.H.) and α -amylase solution (0.3 ml.; 1 unit).

k and l EDTA solution (0.1 ml.; 10^{-1} M) and α -amylase solution (0.3 ml.; 1 unit).

m and n Trypsin (1 mg.; crystalline, B.D.H.) and α -amylase solution (0.3 ml.; 1 unit).

o Control: α -amylase solution (0.3 ml.; 1 unit).

After the digests had been left at 18°C for 18 hours, aqueous calcium chloride solution (1 ml.; M) was added to digests j, l and n, and all digests were left for a further 2 hours at 18°C. Aqueous amylose solution (10 ml.; 0.2%) was added to the mixtures, which were then incubated at 35°C for 24 hours. The digests were heated on a boiling-water bath for 5 minutes, cooled, and the amylose remaining in the digests was precipitated with excess of n-butanol. The viscosities of these amylose-butanol complexes were measured in 0.2 M potassium hydroxide, and the results are given below.

Table 4.05

Digest	i	j	k	l	m	n	o
Conditions	EDTA+ trypsin	EDTA+ trypsin+Ca ⁺⁺	EDTA	EDTA+ Ca ⁺⁺	Trypsin	Trypsin+ Ca ⁺⁺	Control
[η] of amylose	330	190	320	160	130	130	70

[η] of original amylose = 330.

4b. Effect of temperature on α -amylolytic activity

(1) Stability of the enzymes/temperature

Acetate buffer (1 ml.; 0.2 M; pH 5.5) and distilled water (5.5 ml.) were brought to the required temperature, α -amylase solution (0.5 ml.; 1-4 units) was added, and the mixtures were left at this temperature for 60 minutes. The digests were then transferred to an incubator at 35°C, and after 30 minutes, warm amylopectin β -limit dextrin solution (1 ml.; 0.6%) was added to each and the enzymic activities were measured. These activities were expressed as a fraction of the activity in a standard digest (pH 5.5; 35°C) where the enzyme had not been preincubated. The results are shown in Figs. 4.1, 4.3 and 4.5.

The stock solution of α -amylase from wheat contained calcium acetate (0.2%).

(11) Activity of the enzymes/temperature

Digests containing acetate buffer (1 ml.; 0.2 M; pH 5.5), amylopectin β -limit dextrin solution (1 ml.; 0.6%) and distilled water (5.5 ml.) were equilibrated at the required temperature, then enzyme solution (0.5 ml.; 1-4 units) was added, and the α -amylase activities were determined as usual. The activities, expressed as fractions of the maximum activity, are shown in Figs. 4.1, 4.3 and 4.5.

Again the stock wheat α -amylase solution contained calcium acetate (0.2%).

FIG. 4.1

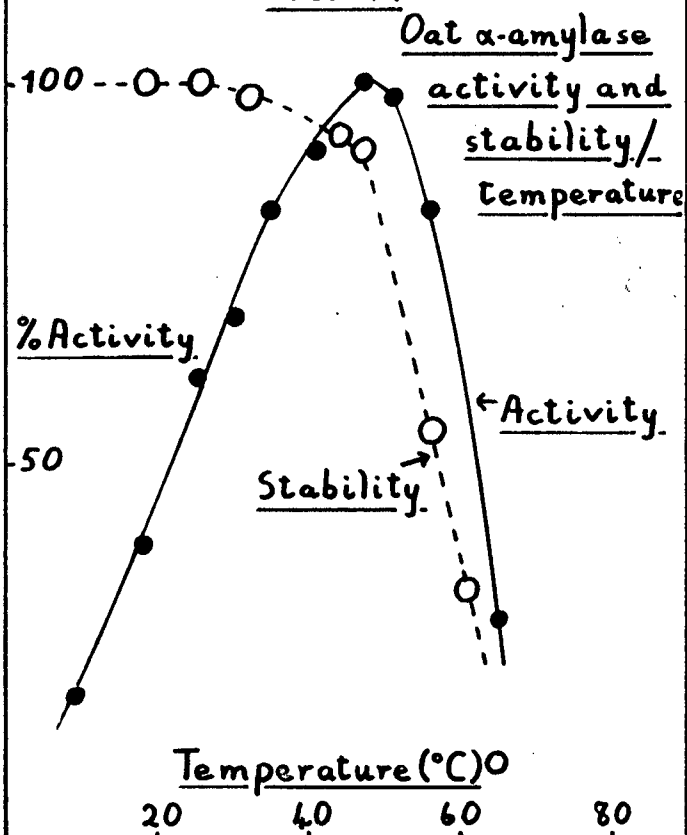


FIG. 4.2

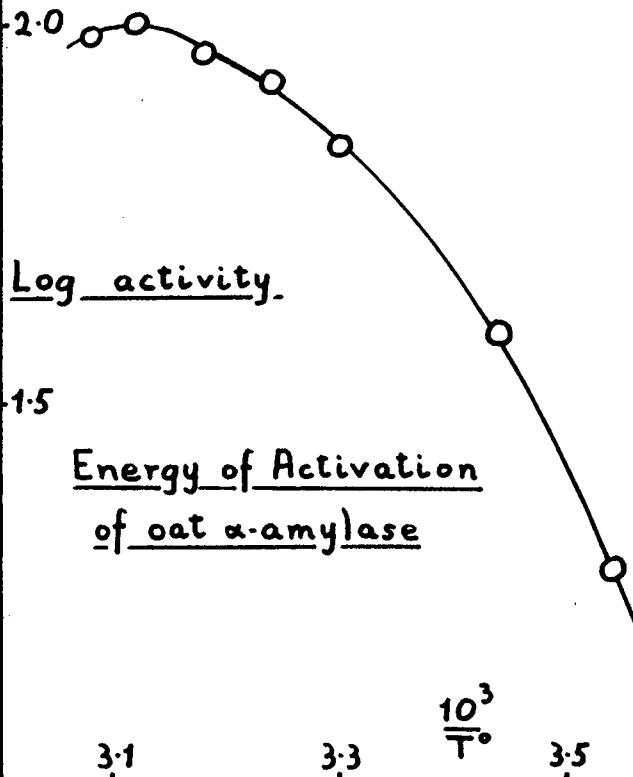


FIG. 4.3

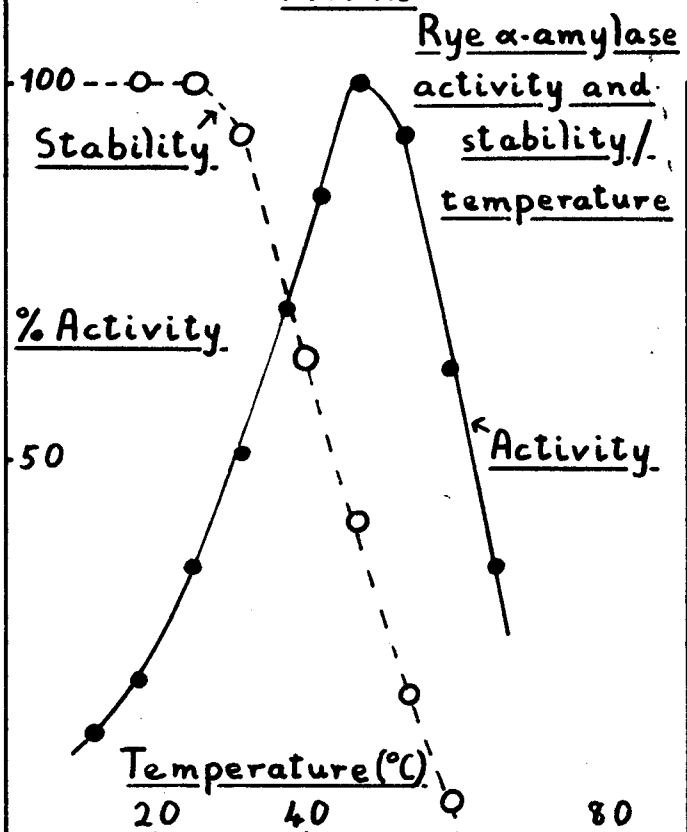
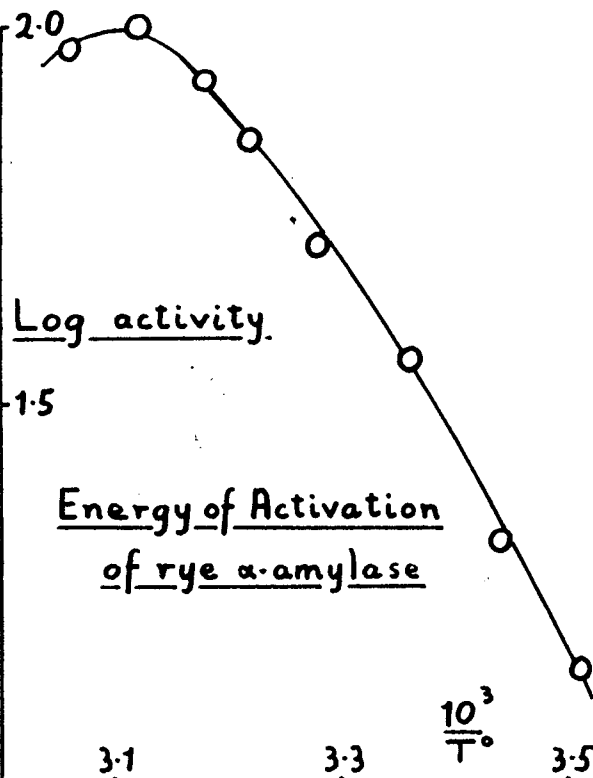


FIG. 4.4



(iii) Energy of activation of the cereal α -amylases

The results from the previous experiments were plotted as the logarithm of the activity at each temperature versus $\frac{10^3}{T}$, where T is the absolute temperature (Figs. 4.2, 4.4 and 4.6). From the slopes of these plots, the apparent activation energies of the enzymic reactions were calculated, and are given in Table 4.06.

Table 4.06

Temperature	Apparent energy of activation (K.cals./mole)		
	Oat α -amylase	Rye α -amylase	Wheat α -amylase
9°C	14	14	14
25°C	9	11	11
Optimum temperature	0	0	0

4c.

Effect of pH

McIlvaine's phosphate-citrate buffers were used in the experiments involving soya-bean α -amylase, while experiments with broad-bean and cereal α -amylases were carried out in disodium hydrogen phosphate-acetic acid buffers (0.2 M).

(i) Stability of the α -amylases/pH

Buffer solution (2 ml.) of the required pH and enzyme solution (1-2 units) were kept at 18°C for 65 minutes (for the cereal α -amylases) or 75 minutes (for the bean α -amylases), then the pH was brought to 5.5 by the addition of dilute

FIG. 4.5

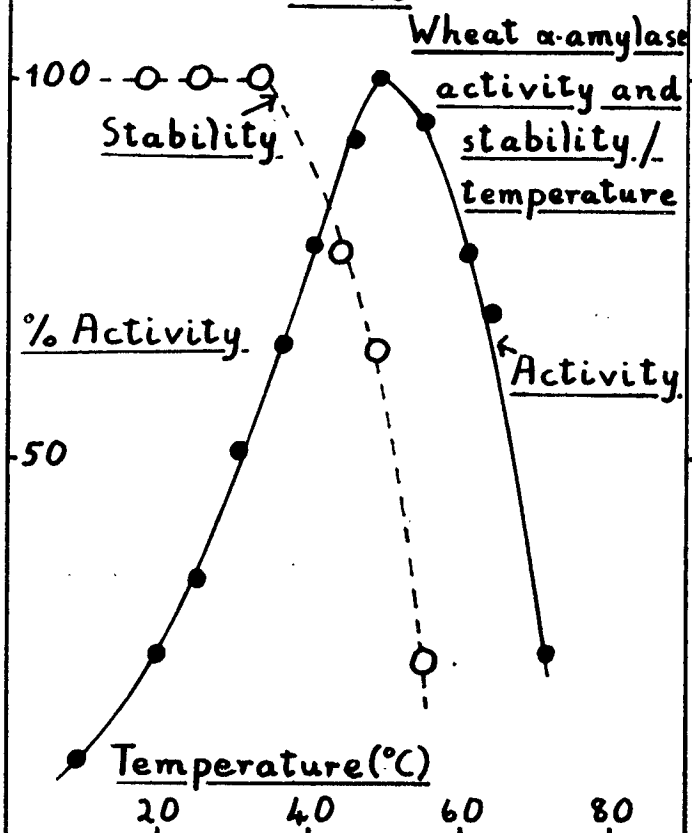


FIG. 4.6

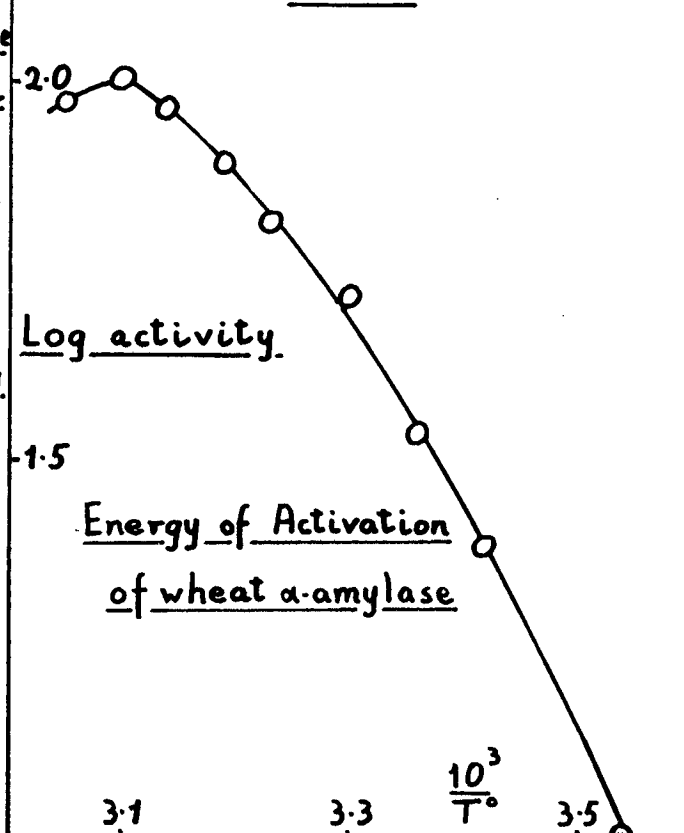


FIG. 4.7

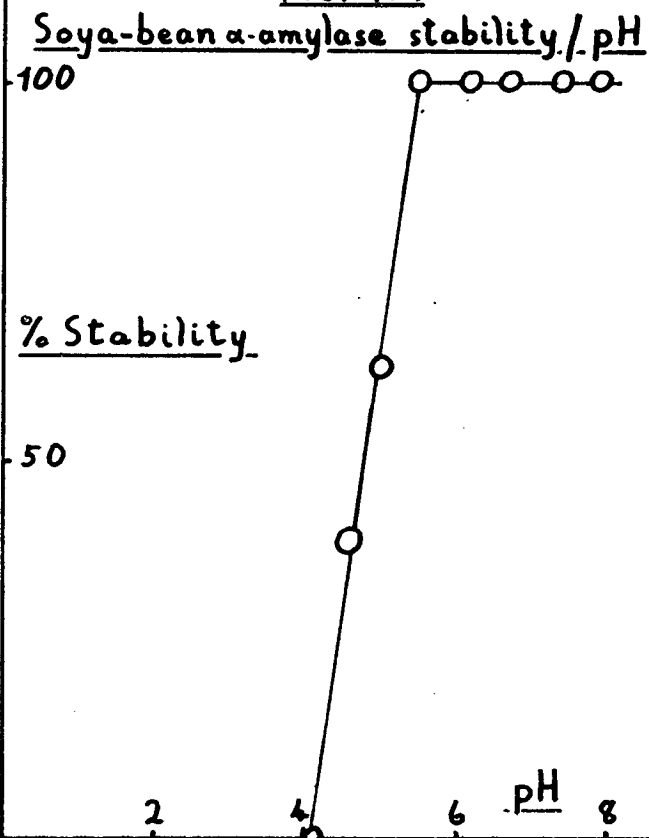
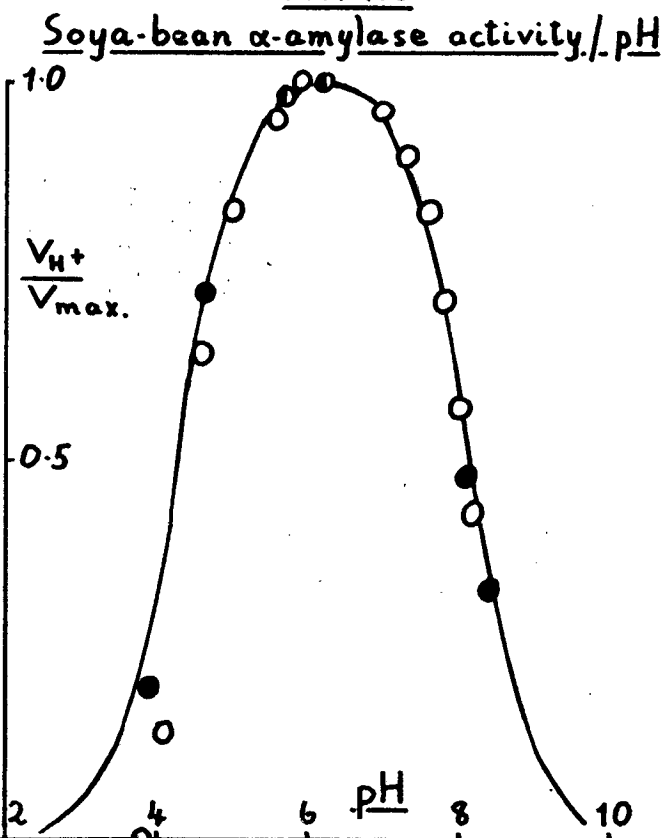


FIG. 4.8



acetic acid or sodium hydroxide solutions, and the mixtures were incubated at 35°C for 20 minutes. Aliquots of amylopectin β -limit dextrin solution (1 ml.; 0.6%; at 35°C) were then added to the digests, the volumes were made up to 10 ml. with warm distilled water (at 35°C) and samples (3 ml.) of the digests were removed at intervals for iodine-staining. Amylolytic activities were calculated in the usual manner, and are shown in Figs. 4.7, 4.9, 4.11, 4.13 and 4.15 as fractions of the activity in a standard digest (pH 5.5; 35°C) where the enzyme had no preincubation in buffer solution.

(11) Activity of the enzymes/pH

Buffer solution (1 ml.) of the appropriate pH, amylopectin β -limit dextrin solution (1 ml.; 0.6%) and distilled water (5.5 or 5.9 ml.) were equilibrated at 35°C, enzyme solution (0.5 or 0.1 ml.; 2 units) was added, and the amylolytic activities were measured. These determinations were repeated with digests containing buffer (1 ml.), substrate solution (4 ml.), distilled water (2.5 or 2.9 ml.) and enzyme solution (0.5 or 0.1 ml.). The results are given in Figs. 4.8, 4.10, 4.12, 4.14 and 4.16, where the enzymic activity, as a fraction of the maximum activity at the optimum pH, is plotted against pH. (-O- represents experimental values obtained with the lower substrate concentration, while -●- represents values obtained with the higher substrate concentration.)

FIG. 4.9

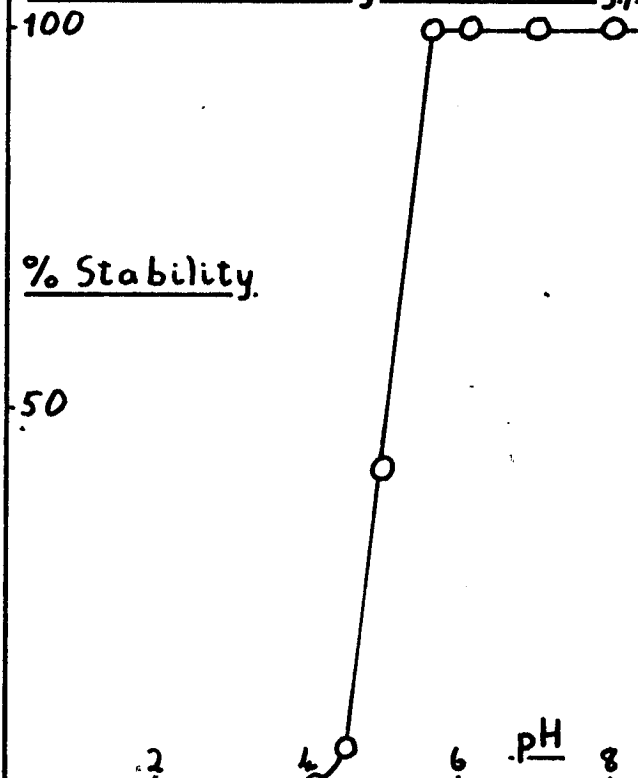
Broad-bean α -amylase stability/.pH

FIG. 4.10

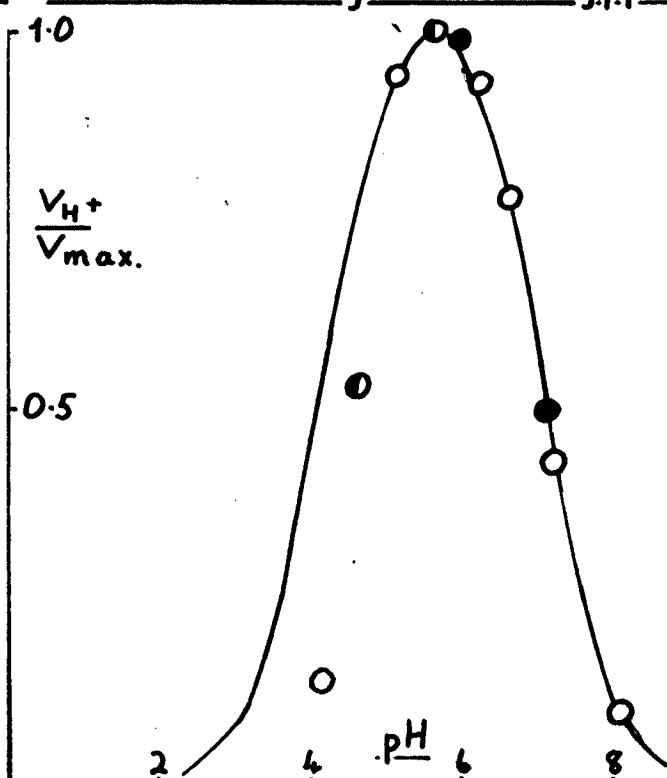
Broad-bean α -amylase activity/.pH

FIG. 4.11

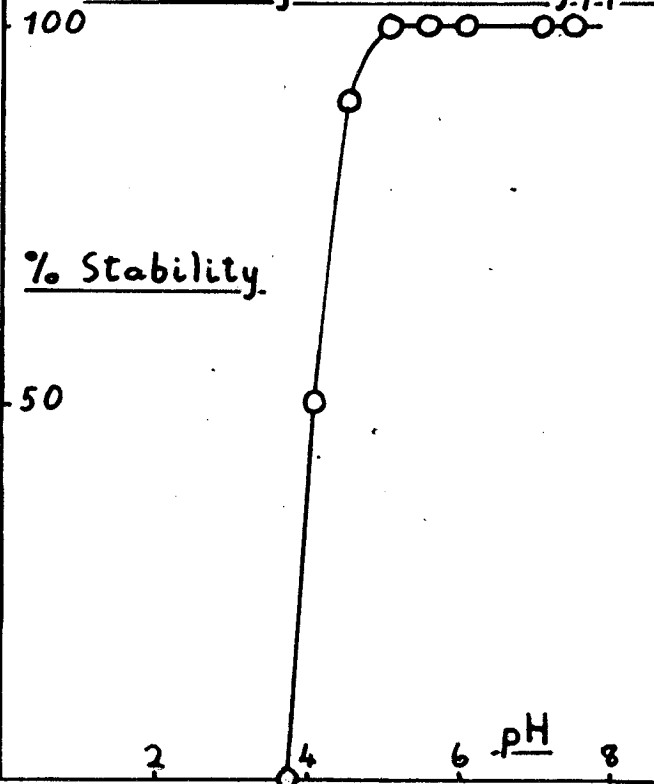
Oat α -amylase stability/.pH

FIG. 4.12

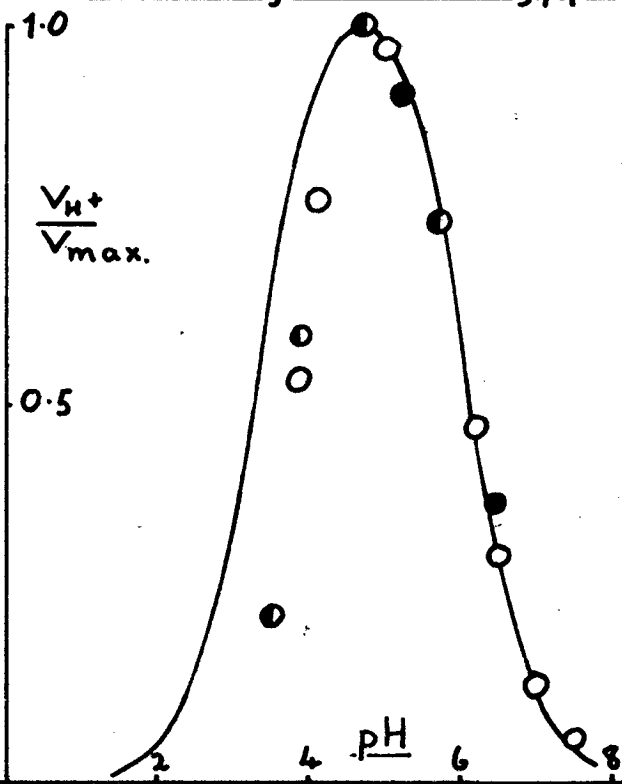
Oat α -amylase activity/.pH

FIG. 4.13

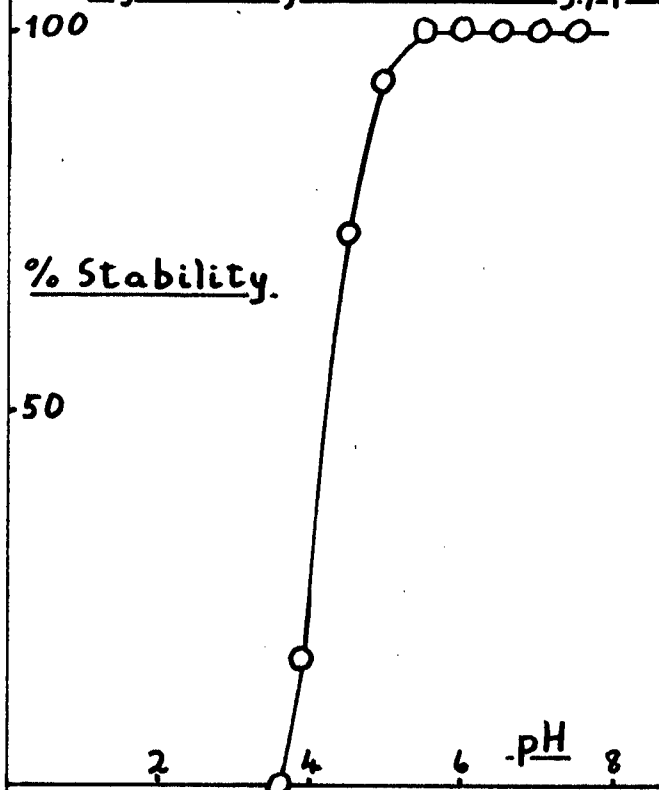
Rye α -amylase stability/pH

FIG. 4.14

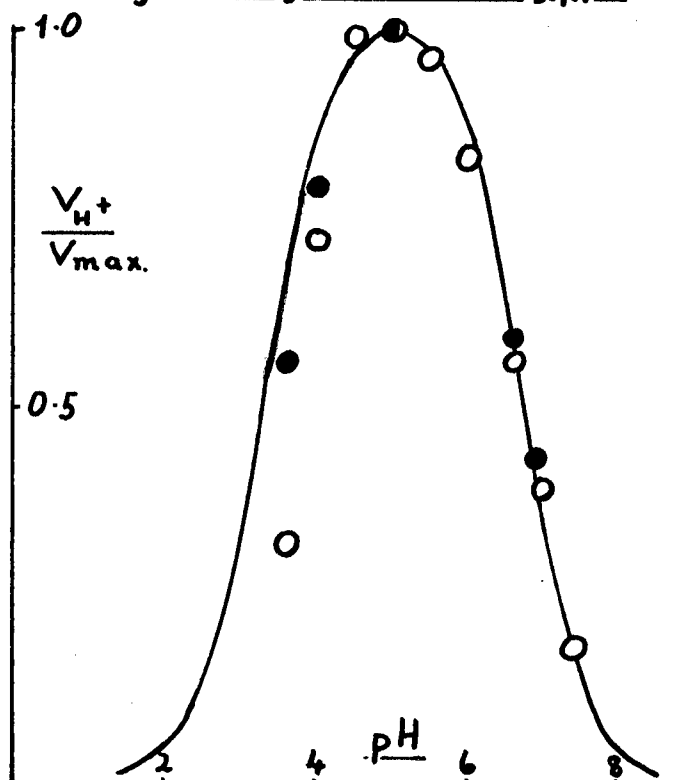
Rye α -amylase activity/pH

FIG. 4.15

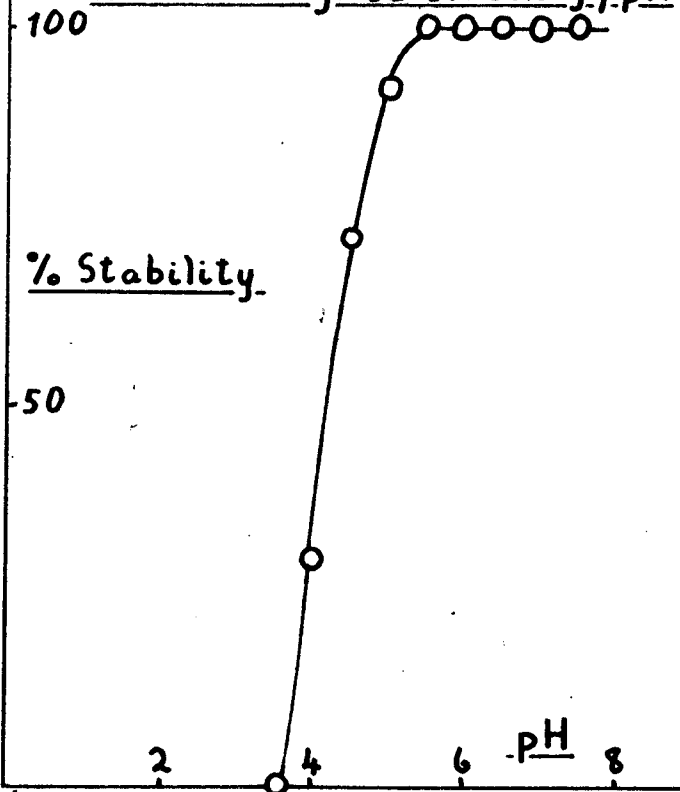
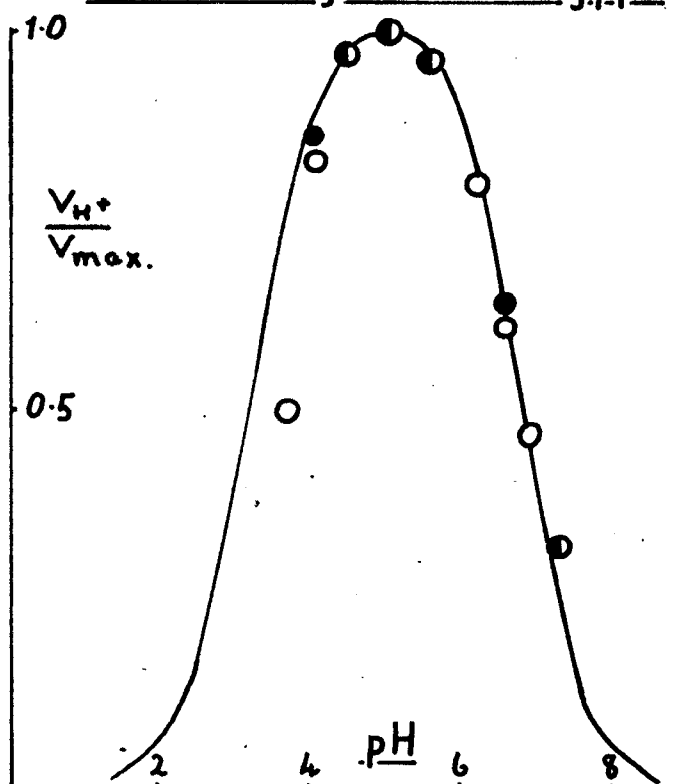
Wheat α -amylase stability/pH

FIG. 4.16

Wheat α -amylase activity/pH

(iii) Ionisation constants of ionisable groups at the active centres of the enzymes

Values of the ionisation constants, K_a and K_b , of groups at the enzyme active centres were calculated from the preceding results and the equation

$$\frac{V_{H^+}}{V_{\max.}} = \frac{1 + 2 \sqrt{\frac{K_a}{K_b}}}{1 + \frac{K_a}{[H^+]} + \frac{[H^+]}{K_b}}$$

where V_{H^+} and $V_{\max.}$ represent the rates of enzyme action at hydrogen ion concentration $[H^+]$ and at the optimum pH, respectively (See Discussion). Table 4.07 shows the values obtained.

Table 4.07

Source of α -amylase	Soya-beans	Broad-beans	Oats	Rye	Wheat
$pK_a = -\log_{10} K_a$	8.15	7.1	6.1	6.7	6.8
Maximum possible value of $pK_b = -\log_{10} K_b$	4.3	4.1	3.3	3.3	3.2

The solid lines in Figs. 4.8, 4.10, 4.12, 4.14 and 4.16 represent the function $\frac{V_{H^+}}{V_{\max.}}$ using the values of K_a and K_b in Table 4.07.

(iv) Effect of preincubation at pH 3.6

For the rye and wheat enzymes, digests were prepared as follows:

- p Acetate buffer (2 ml.; 0.2 M; pH 5.5), enzyme (0.2 ml. containing 2 units of rye α -amylase or 0.1 ml. containing 3.5 units of wheat amylase) and amylose solution (10 ml.; 0.2%).
- q Acetate buffer (2 ml.; 0.2 M; pH 3.6), enzyme (as for p) and amylose solution (10 ml.; 0.2%).
- r Acetate buffer (2 ml.; 0.2 M; pH 3.6), enzyme (as for p); incubated for 2 hours at 18°C before amylose solution (10 ml.; 0.2%) was added.
- s Acetate buffer (2 ml.; 0.2 M; pH 3.6), enzyme (0.3 ml. containing 10.5 units wheat α -amylase); incubated for 2 hours at 18°C before addition of amylose solution (10 ml.; 0.2%).

The digests were incubated at 35°C for 25 hours, heated on a boiling-water bath for 5 minutes and cooled. Excess of n-butanol was added to the mixtures to precipitate the amylose products. No precipitate appeared in digest p. The viscosities of the amylose-butanol complexes were measured in 0.2 M potassium hydroxide, and were as given in Table 4.08.

Table 4.08

Digest	p	q	r	s	
Conditions	pH5.5	pH3.6 directly	pH3.6 prein- cubation	pH3.6 high enzyme concentration; * preincubation	Original amylose
[η] of amylose + rye α -amylase	<30	130	300	n.d.	330
[η] of amylose + wheat α -amylase	<30	110	190	50	210

n.d. \equiv not determined.

* enzyme concentration in s was three times that in r
(See Experimental).

4d.

Modification of the α -amylases

from soya- and broad-beans, oats,

rye and wheat. [Fraenkel-Conrat (1957)].

(1) Iodination

Digests containing acetate buffer (1 ml.; 0.2 M; pH 5.5), iodine solution (0.2 ml.; $1.2 \times 10^{-4}M$) and distilled water (5.3 ml.) were equilibrated at 35°C, then enzyme solution (0.5 ml.; 1-5 units) was added. After 15 minutes, aliquots of amylopectin β -limit dextrin solution (1 ml.; 0.6%; at 35°C) were added, and the α -amylolytic activities were determined.

All the enzymes studied were completely inhibited by this treatment.

(ii) Coupling with diazobenzenesulphonic acid

Phosphate buffer (1 ml.; 0.2 M; pH 8) and p-diazobenzenesulphonic acid solution (0.1-0.3 ml.; 0.6%) were added to enzyme solution (2 ml.; 4-6 units); a control was prepared containing sulphanilic acid (0.1-0.3 ml.; 0.6%). The mixtures were left at 18°C for 1-1½ hours, dialysed at 2°C against aqueous calcium acetate solution (0.2%; 3 x 200 ml.) and centrifuged. Enzymic activities were determined in the usual manner, and the results are shown in Table 4.09.

Table 4.09

Source of α -amylase	Soya-beans	Broad-beans	Oats	Rye	Wheat
Percentage inhibition by diazo-acid	95	70	80	90	90

(iii) Acetylation

Sodium acetate (250 mg.) and acetic anhydride (0.03 ml.) were added to enzyme solution (1 ml.; 2-6 units) at 0°C; a control digest contained no anhydride. After 1-1½ hours at 0°C, the mixtures were dialysed at 2°C against aqueous calcium acetate solution (0.2%; 3 x 200 ml.). The amylolytic activities of these mixtures were measured as usual, and the results are given in Table 4.10.

Table 4.10

Source of α -amylase	Soya-beans	Broad-beans	Oats	Rye	Wheat
Percentage inhibition by acetic anhydride	75	45	60	25	20

(iv) Effect of sodium p-chloromercuribenzoate

A digest was prepared containing acetate buffer (1 ml.; 0.2 M; pH 5.5), sodium p-chloromercuribenzoate solution (1 ml.; 2.5×10^{-4} M), soya-bean enzyme solution (0.025 ml.; 2 units) and distilled water (5 ml.). The mixture was left at 18°C for 2 hours, then at 35°C for 30 minutes, amylopectin β -limit dextrin solution was added (1 ml.; 0.6%; at 35°C), and the enzymic activity was determined.

The enzyme had the same activity as that in a control digest containing no mercuribenzoate.

A second digest was prepared as above, using 0.2 ml. broad-bean enzyme solution (3 units) and 4.8 ml. water. The mixture was incubated for 15 minutes at 35°C before the addition of dextrin. The amylolytic activity was measured over a period of 30 minutes, and was found to be the same as that in a control digest.

However, when a digest containing acetate buffer (1 ml.; 0.2 M; pH 5.5), sodium p-chloromercuribenzoate solution (5 ml.; 8×10^{-5} M), broad-bean enzyme solution (0.5 ml.; 3 units) and distilled water (0.5 ml.) was incubated at

35°C for 2 hours before the addition of warm dextrin solution (1 ml.; 0.6%), the enzyme was found, after a further 70 minutes, to have retained only 30% of the activity of the enzyme in a control digest.

For the cereal α -amylases digests were set up as follows:

t Sodium acetate buffer (1 ml.; 0.2 M; pH 5.5), enzyme solution (0.5 ml.; 1-3 units) and distilled water (5.5 ml.).

u Sodium acetate buffer (as for t), enzyme solution (as for t), distilled water (5.4 ml.) and sodium p-chloromercuribenzoate solution (0.1 ml.; 8×10^{-5} M).

y and w as t and u, using calcium acetate buffer (1 ml.; 0.1 M; pH 5.5).

These digests were left at 35°C for 90 minutes, amylopectin β -limit dextrin solution (1 ml.; 0.6%; at 35°C) was added, and the enzymic activities were measured as usual. The results are shown below.

Table 4.11

Source of α -amylase	Oats		Rye	Wheat
Time of testing activity after addition of dextrin (minutes)	50	120	50	50
% Inhibition (sodium acetate buffer)	50	65	70	100
% Inhibition (calcium acetate buffer)	30	45	30	60

Further digests were prepared containing sodium acetate buffer (1 ml.; 0.2 M; pH 5.5), sodium p-chloromercuribenzoate solution (6 ml.; $8 \times 10^{-5} \text{M}$), and hog pancreas or Bacillus subtilis α -amylase solution (0.025 ml.; 5 units). After standing at 35°C for 2 hours, warm dextrin solution (1 ml.; 0.6%) was added to the digests and the amylolytic activities were determined.

In both cases, the enzyme had the same activity as that in a control digest without mercuribenzoate.

4e. Molecular size of the α -amylases,
determined by gel filtration

A column (33 cm. x 1.25 cm.) of Bio-Gel P-100, equilibrated in acetate buffer (0.2 M; pH 5.7) containing calcium acetate (0.2%), was prepared.

Mannitol (30 mg.) was added to dialysed enzyme solution (0.5 ml.; 100 units malted cereal α -amylase or 20 units wheat α -amylase) in acetate buffer, to increase the density of the mixture. The sample was then injected onto the top of the column, and the enzyme was eluted from the gel with acetate buffer, using a constant-flow pump to regulate the flow-rate (at 8 ml./hour). The eluate was collected in 0.6 ml. aliquots by means of an L.K.B. "Radirac" fraction collector. (I wish to thank Dr. R. Wall for the use of this equipment.) The α -amylase activity of each sample was measured.

The void volume of the column was determined by finding the elution volume of Blue Dextran 2000 (Pharmacia, Uppsala; molecular weight ca. 2×10^6).

The ratio $\frac{\text{elution volume of } \alpha\text{-amylase (V}_e\text{)}}{\text{column void volume (V}_o\text{)}}$ was calculated for each enzyme and the results are shown below.

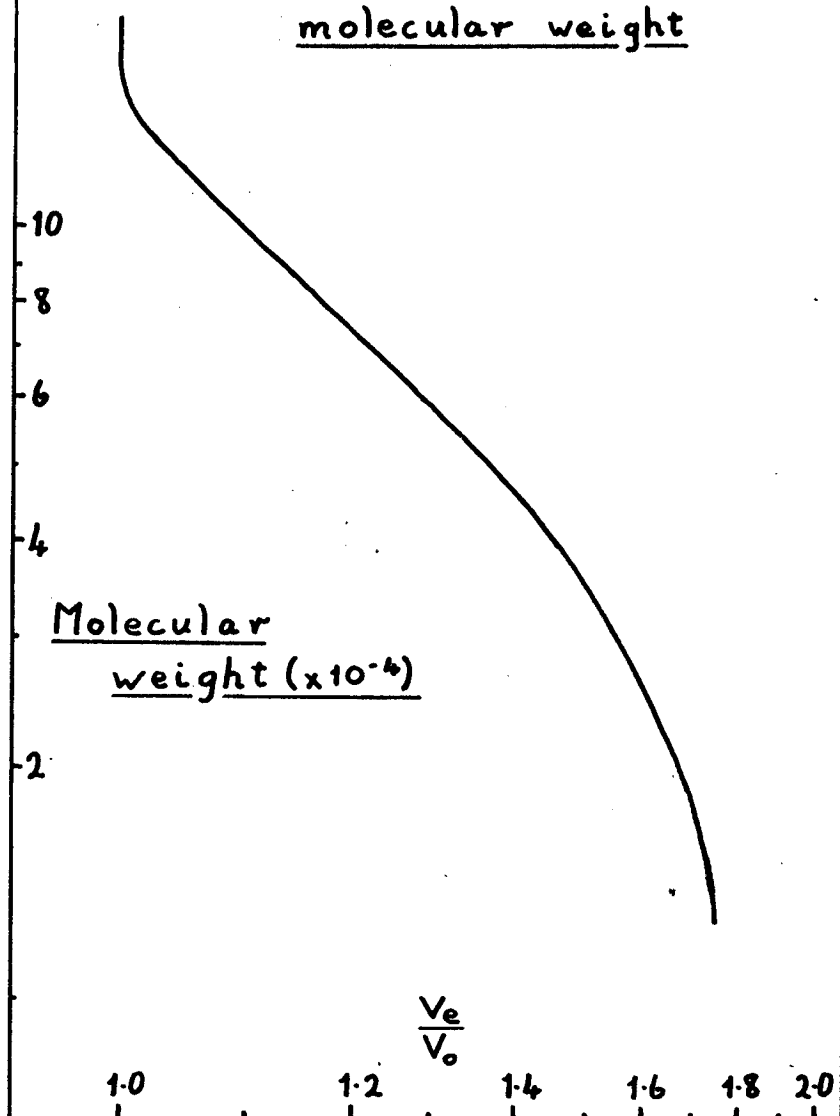
Table 4.12

Enzyme	$\frac{V_e}{V_o}$
Malted barley α -amylase	1.40 ± 0.05
Malted wheat α -amylase	1.39 ± 0.05
Wheat α -amylase	1.36 ± 0.05

Using a graph of molecular weight versus $\frac{V_e}{V_o}$ (Fig. 4.17) reproduced from "Materials for Ion Exchange, Adsorption and Gel Filtration" (Bio-Rad Laboratories publication, May, 1965) the molecular weight of the three enzymes was estimated to be $50,000 \pm 5,000$.

FIG. 4.17

Variation of elution volume with
molecular weight



Discussion

Effect of various reagents on α -amylase activity: It was shown (Table 4.01) that $10^{-3}M$ solutions of sodium chloride, potassium cyanide, potassium nitrate and potassium sulphate had no effect on the activity. Also, provided that the α -amylase solutions contained sufficient calcium to stabilise the enzymes additional calcium chloride ($10^{-3}M$) produced no activation. Similar results were obtained by MacGregor (1964) for soya- and broad-bean, barley and malted barley α -amylases.

Ammonium molybdate ($10^{-3}M$) strongly inhibited the enzymes, but more dilute solutions ($10^{-4}M$) had no effect. MacGregor (1964) reported 20-30% inhibition of bean and barley amylases by $10^{-3}M$ ammonium molybdate.

Silver and mercuric ions were very efficient inhibitors, while lead and copper were less effective (Table 4.02); inhibition was caused by the cations rather than the anions as shown by the lack of effect of the corresponding sodium and potassium salts (Table 4.01). The oat enzyme was less susceptible to silver and mercury ions than the rye and wheat amylases.

The inhibiting action of heavy metals on plant α -amylases has been known for many years; Olsson (1921) and Myrbäck and Frostell (1946) reported that silver ions inactivated malt α -amylase. Later, Stewart (1963) found that mercury and copper slowed down the reaction of sorghum amylase.

The mechanism of this inhibition is still uncertain. Some workers have suggested that heavy metals react with sulphydryl groups in enzymes, but the α -amylase of B. subtilis, which does not contain such groups [Junge et al. (1959)], has been shown to be inhibited by silver, mercury, lead and copper [Di Carlo and Redfern (1947), Urata (1957)]. Myrbäck (1957) concluded that silver inhibited a yeast fructofuranosidase by combination with a histidine residue in the enzyme molecule. As histidine is considered important for the activity of α -amylases [Ono et al. (1958), Thoma et al. (1963)], such combination may account for the effect of heavy metals on the amylases.

Phosphate-citrate buffer decreased the activity of the cereal and broad-bean α -amylases, but not that of the soya-bean enzyme (p. 55 and 61). It is likely that the effect of McIlvaine's buffer is to remove calcium ions from the α -amylases, thus rendering them inactive. Citrate or phosphate ions separately had little effect (10% for citrate, none for phosphate) on broad-bean amylase activity; only when the two types of ion acted together was their calcium-binding action strong enough to deactivate the enzyme. The smaller inhibition of the wheat α -amylase (in calcium acetate solution) compared to that of the rye enzyme (20% and 50%, respectively) showed that the metal ion was capable of protecting the amylases against the effects of McIlvaine's buffer. It seems probable that calcium ions are bound rather more tightly

to the soya α -amylase than to the other plant amylases studied, for this enzyme was stable for 2 hours in phosphate-citrate buffer at pH values greater than 5.5 (p. 61).

Ascorbic acid (10^{-3}M) completely inhibited the cereal enzymes, but at greater dilutions the effect was decreased, and no inhibition was observed in digests containing 10^{-5}M acid (Table 4.03). MacGregor (1964) found that ascorbic acid (10^{-3}M) produced 93 and 100% deactivation of malted barley and soya-bean α -amylases, respectively.

Larner and Gillespie (1956) reported that tris buffer [2-amino-2-(hydroxymethyl)-propane-1:3-diol; $2 \times 10^{-4}\text{M}$] decreased the activity of an intestinal maltase by competing with the substrate for the enzyme. However, no inhibition of the cereal α -amylases was observed in digests containing 10^{-3}M tris (Table 4.03).

Ethylene diamine tetraacetate, acting alone or in the presence of trypsin, completely deactivated the α -amylases studied (Table 4.04, digests a and c). This loss of activity is thought to be due to the removal of calcium ions from the enzymes and was partially reversed by the addition of calcium (digests b and d). Other plant α -amylases have been shown to react similarly; Fischer and Haselbach (1951) showed that 80% of the activity of germinated barley α -amylase (deactivated by EDTA) could be recovered on dialysis against calcium, while MacGregor

(1964) described the partial reversal of EDTA-inhibition by added calcium for the amylases of soya- and broad-beans. When trypsin, as well as EDTA, was present in the digests (digest b), rather more irreversible deactivation of the oat and wheat enzymes was obtained, than when the protease was absent (digest d). This would be expected on the basis of the results of Stein and Fischer (1958), who found that removal of calcium from α -amylases renders them susceptible to proteolytic attack. They further concluded that, if little activity was recovered on the addition of calcium ions to an EDTA-deactivated amylase, then proteases were present in the α -amylase preparations.

Thus the amylases from oats and wheat may contain proteolytic enzymes, while the rye preparation may be relatively free from these contaminants.

The inhibition produced by trypsin alone for cereal α -amylases is surprising (Table 4.04, digest e), as α -amylases are generally resistant to proteolytic attack. However, MacGregor (1964) has reported slight inhibition of soya- and broad-bean amylases by trypsin. The calcium-protein binding may be so weak for these plant enzymes that, even in the absence of chelating agents, some degradation by trypsin can take place.

It is known that calcium exerts a stabilising influence on both trypsin and α -amylases [Bier and Nord (1951)], and in this case (Table 4.04, digest f) addition of an excess of calcium stabilised the protease more than the amylases; thus the inhibition of the α -amylases was increased.

Measurement of amylose-viscosities provides a more sensitive method of studying α -amylolytic attack. This technique was used for the rye amylase in an attempt to determine whether the effect of EDTA was completely reversible. The results (Table 4.05, digests j and l) showed that the inhibition was not fully reversed on the addition of calcium and that irreversible deactivation of the α -amylase was greater in the presence of EDTA and trypsin, than with EDTA alone. Further, it is possible that there were small quantities of active protease present in the rye amylase preparation (too small to be detected under the conditions of digests a-f). Trypsin alone had some effect on the α -amylase, but this inhibition was unchanged by the addition of calcium (digests m and n). As trypsin is unstable in aqueous solutions [Bier and Nord (1951)], it seems likely that, in digests m and n, the trypsin was inactive after 18 hours, and so the added calcium could have no effect on the tryptic digestion of the amylase.

Effect of temperature: The cereal α -amylases studied exhibited maximum activity in the temperature range 47-49°C (Figs. 4.1, 4.3 and 4.5). These results are in good agreement with observations of Greenwood and MacGregor (1965) who found that the optimum temperature for barley and malted barley amylases was in the range 45-50°C. Fischer and Haselbach (1951), however, reported that the temperature range of maximum activity for the malted

barley enzyme was 50-55°C. The two sets of results are not incompatible, for the value obtained for an optimum temperature is governed by the rate of denaturation of the α -amylase in the test solutions. As the amount of heat-denaturation undergone by the amylase depends on the concentration of calcium, inert protein and even of the amylase itself, as well as on the time taken to measure the activity, variations of ca. 5°C between values obtained by different workers might be expected.

Stability experiments showed that the α -amylases completely retained their activity after one hour's incubation at 25°C, and lost only 10% of the original activity at 46°C (oat enzyme), 32°C (rye enzyme) and 40°C (wheat enzyme). There was a rapid decrease in the stability of each amylase at temperatures above those mentioned (Figs. 4.1, 4.3 and 4.5).

As the specific activity of the oat enzyme (51 units/mg./ml.) was lower than those of the other amylases studied (410 and 260 units/mg./ml. for the rye and wheat enzymes, respectively), it is probable that there was much inert protein in this preparation, and this material would be expected to protect the α -amylase against heat denaturation. Also, the wheat enzyme solution contained a relatively high concentration of calcium ions capable of increasing the heat-stability of the α -amylase. Thus it is not surprising that both the oat and wheat amylases were more stable than that of rye.

In the stability experiments the amylases were very rapidly denatured at 65°C, while during the preparation of the enzymes (Section 3b, c and d) ca. 40% of the α -amylolytic activity was recovered after heating at 70°C for 20 minutes. The results are not contradictory, for purified enzymes were used for the stability measurements, and these solutions contained much less protective protein than the crude fractions heated to deactivate β -amylase.

Values of the apparent activation energy of the overall enzyme reaction were calculated from graphs (Figs. 4.2, 4.4 and 4.6) of the Arrhenius relation

$$\log a = \frac{-E}{RT} + C,$$

where a represents the amylase activity, E the activation energy, R the gas constant, T the absolute temperature and C is a constant.

The activation energy for the cereal amylases was 14 K. calories/mole at 9°C and 9-11 K. calories/mole at 25°C. These are similar to values obtained for other plant α -amylases by MacGregor (1964) i.e. 14 and 13 K. calories/mole at 9°C for the enzymes from beans (soya- and broad-beans) and cereals (barley and malted barley), respectively.

These values are much lower than activation energies of non-enzymic hydrolyses of similar polysaccharides e.g. for acid hydrolysis the activation energy is ca. 30 K. calories/mole [French (1957)].

One possible explanation of this effect is that the enzyme attacks a strained form of the substrate, so that relatively little additional energy is required to raise the amylase-substrate complex to the fully-activated state where hydrolysis occurs.

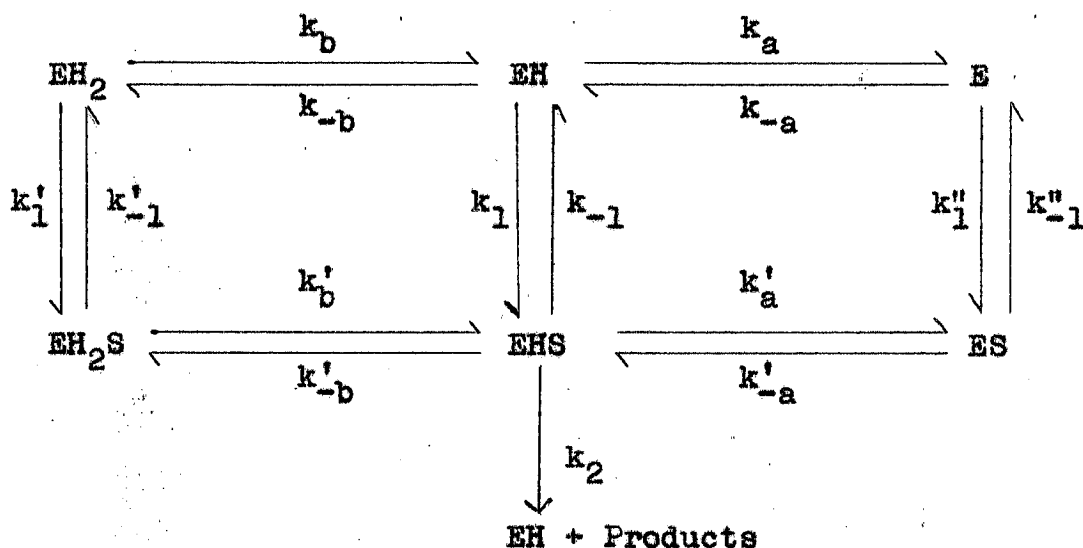
Effect of pH: All the α -amylases studied were stable for one hour at 18°C at pH values between 5.5 and 7.5; the soya- and broad-bean enzymes were rapidly and irreversibly inactivated below pH 5.5, while, for the cereal enzymes, the loss of activity was rapid only below pH 5.0 (Figs. 4.7, 4.9, 4.11, 4.13 and 4.15).

The stability of the cereal amylases is similar to that found for the α -amylase of malted barley by Fischer and Haselbach (1951). It must be stressed, however, that the stability of an α -amylase is very much influenced by the presence of calcium and inert protein.

The pH ranges of maximum activity were 6.0-6.4 for the soya α -amylase, 5.6 for the broad-bean enzyme and 4.7-5.0 for the cereal amylases (Figs. 4.8, 4.10, 4.12, 4.14 and 4.16). The values for the oat, wheat and rye enzymes are similar to those obtained by Fischer and Haselbach (1951). They reported that the most favourable pH range for malted barley α -amylase was 4.7-5.4. A value of pH 5.5 for the optimum pH of germinated rye α -amylase was found by Ohlsson and Uddenberg (1933). This is higher than the value found here for the amylase of ungerminated rye, but the discrepancy is probably due to differences in the stabilities of the enzymes used.

The shapes and positions of the pH/activity curves (Figs. 4.8, 4.10, 4.12, 4.14 and 4.16) were independent of substrate concentration.

The enzymes were assumed to contain two ionisable, reactive groupings, and the following hypothetical scheme was used to analyse the results [Waley (1953), Alberty and Massey (1954), Laidler (1955), Ono et al. (1958)]:



where E = enzyme, H = proton, S = substrate and k = velocity constants as shown.

The ionisation constants of the enzyme (K_a , K_b) and the enzyme-substrate complex (K'_a , K'_b) are defined as

$$K_a = \frac{k_a}{k_{-a}}, \quad K_b = \frac{k_b}{k_{-b}}, \quad K'_a = \frac{k'_a}{k'_{-a}}, \quad K'_b = \frac{k'_b}{k'_{-b}}.$$

If it is assumed that (i) the form EHS is the only complex capable of breaking down to give the reaction products and (ii) the ionisation of the two groups is unaffected by substrate binding, so that $K_a = K'_a$ and

$K_b = K'_b$, then the rate of the enzymic reaction (V_{H^+}) at hydrogen ion concentration $[H^+]$ may be expressed as

$$V_{H^+} = \frac{k_2[E][S]}{(1 + \frac{K_a}{[H^+]} + \frac{[H^+]}{K_b})(K_m + [S])}, \quad 1.$$

where K_m is the Michaelis constant ($K_m = \frac{k_{-1}}{k_1}$), and $[E]$ and $[S]$ represent the enzyme and substrate concentrations, respectively.

Differentiation of equation 1. with respect to $[H^+]$, to find the hydrogen ion concentration, $[H^+]_{opt.}$, at which V_{H^+} is a maximum, gives

$$[H^+]_{opt.} = \sqrt{K_a K_b} \quad 2.$$

Then from equations 1. and 2., the maximum value of V_{H^+} ($V_{max.}$) is given by

$$V_{max.} = \frac{k_2[E][S]}{(1 + 2\sqrt{\frac{K_a}{K_b}})(K_m + [S])} \quad 3.$$

Then the ratio $\frac{V_{H^+}}{V_{max.}}$ is represented by (from equations 1. and 3.)

$$\frac{V_{H^+}}{V_{max.}} = \frac{1 + 2\sqrt{\frac{K_a}{K_b}}}{1 + \frac{K_a}{[H^+]} + \frac{[H^+]}{K_b}} \quad 4.$$

The equation indicates that $\frac{V_{H^+}}{V_{max.}}$ is independent of substrate concentration. As the results in Figs. 4.8, 4.10, 4.12, 4.14 and 4.16 showed such an independence, this

function was used to calculate K_a and K_b (p. 63).

Values for pK_a were 8.15 for soya-bean α -amylase, 7.1 for broad-bean amylase and 6.1-6.8 for the cereal enzymes (Table 4.07). For the soya-bean enzyme, a value of $pK_b = 4.3$ was obtained. Because of the instability of the α -amylases at high hydrogen-ion concentrations, determination of pK_b for the other enzymes studied was less accurate. For these amylases, pK_b was calculated from equation 2., and was found to be 4.1 for the broad-bean α -amylase, and 3.2-3.3 for the cereal enzymes. These figures represent the maximum possible values of pK_b for the α -amylases, as the maximum activity might be found at a lower pH, if the effects of the instability of the enzymes could be eliminated.

The solid line in Figs. 4.8, 4.10, 4.12, 4.14 and 4.16 represents the function $\frac{V_{H^+}}{V_{max}}$ for the values of K_a and K_b in Table 4.07. The experimental points lie on this line, at hydrogen-ion concentrations where the enzymes were stable; at lower pH values the experimental points lie below the line.

The nature of the ionising groups in the amylases may be inferred from the calculated pK values. The group of $pK_b = 3.2-4.3$ is most likely a carboxylic acid (as found in glutamic or aspartic acids), while that of $pK_a = 6.1-7.1$ is probably the imidazolium grouping of histidine [Edsall (1943)]. The group in the soya-bean enzyme of $pK_a = 8.15$ may correspond to an amino group. There is, however,

the possibility that interaction with an anion may have displaced the pH/activity curve [Myrbäck (1926)] and in this case the unknown ionising group may be an imidazolium ring.

The behaviour of the enzymes with respect to pH has been explained on the basis of the assumption that the ionisation of the two groups is unaffected by substrate binding. It is probable, then, that these groups are involved in the breakdown of the amylase-substrate complex (to give the reaction products) rather than in its formation.

By studying the effect of pH on α -amylolytic activity, several workers [Ono et al. (1958): bacterial amylase, Thoma et al. (1963): mammalian amylase, Dube (1961): plant amylase] have concluded that carboxyl and imidazolium groups form part of the enzymic active centres.

The effect of preincubation at pH 3.6 on the rye and wheat enzymes was studied by following changes in the viscosity of an amylose (Table 4.08). The results show that extensive degradation of the amylose was caused by the enzymes acting directly at pH 3.6 (digest q). When the amylases were pretreated at pH 3.6 before addition of the substrate, there was little change in the amylose viscosity, indicating that nearly all the enzymic activity was lost (Compare digest r and the original substrate.). However, there was a significant decrease in the viscosity of amylose to which was added a pretreated, but more concentrated, solution of wheat α -amylase (digest s).

Chromatographic examination of the supernatant solution (from which the amylose-butanol complex had been precipitated) indicated that the decrease was caused by α -amylolytic attack on the substrate and not by another carbohydrase contaminant; traces of small oligosaccharides, $DP \approx 6$, were found on the chromatogram.

It must be concluded, then, that the extent of deactivation of wheat α -amylase by incubation at pH 3.6, in the absence of substrate, depends on the enzyme concentration. Similar observations have been reported by Greenwood and MacGregor (1965) for the α -amylase of malted barley.

Modification of the α -amylases [Fraenkel-Conrat (1957)]:

The enzymes were modified by various reagents in an attempt to gain further information about the nature of the active centres.

The five α -amylases tested (from soya- and broad-beans, oats, wheat and rye) were completely inhibited by $3 \times 10^{-6}M$ iodine (p. 65). As iodine reacts preferentially with imidazolium, phenol or sulphhydryl groups, this suggests that at least one of such groups is necessary for enzymic activity. Reaction with p-diazobenzenesulphonic acid, which couples with tyrosine or histidine residues in proteins, inhibited the α -amylases by 70-95% (Table 4.09), confirming that phenol and/or imidazolium groupings form an important part of the enzyme active centre.

The activity of the amylases was reduced by 20-75% (Table 4.10) by acetic anhydride. This reagent is reasonably specific for free amino groups, but will also attack sulphhydryl and phenolic hydroxyl groups. As there was insufficient enzyme available for a structural investigation of the types of groups acetylated, it is not possible to decide on this evidence alone, which of the three types of grouping is most important for amylolytic activity.

Considering these results and the effects of pH discussed above, it seems very likely that imidazolium rings are necessary for the activity of plant α -amylases.

Several workers have concluded that amino groups are involved in the formation of the α -amylase-substrate complex for fungal, bacterial and mammalian enzymes [Radichevich et al. (1959), Benner and Myrbäck (1952), Little and Caldwell (1942 and 1943)], so that it seems probable that these groups are also important in plant amylases.

There is disagreement concerning the part played by tyrosine residues in α -amylases. Little and Caldwell (1942), Radichevich et al. (1959) and Thoma et al. (1963) consider that these residues are not necessary for activity, while Yamamoto (1955) and Ikenaka (1959) believe that tyrosine is important. Takagi and Isemura (1960 and 1961), on the other hand, have put forward the theory that phenolic hydroxyl groups participate in

maintaining the stability of the enzyme molecules. From the results obtained in this work, it is not possible to draw any conclusions concerning the role of tyrosine in plant α -amylases.

Sodium p-chloromercuribenzoate is generally considered to react specifically with sulphydryl groups. Under the conditions used, the soya-bean, hog pancreas and B. subtilis enzymes were unaffected (p. 67); thus it is very likely that sulphydryl groups are not involved in the active centres of these enzymes. The reagent had no immediate effect on broad-bean α -amylase, but at a higher concentration and with two hours' preincubation, the enzyme was partly inhibited. The cereal enzymes were all inhibited by the p-chloromercuribenzoate, the extent of inhibition depending on the length of time for which reagent and enzyme had been in contact, and on the concentration of calcium ions in the solutions (Table 4.11).

The inhibition of a cereal α -amylase by p-chloromercuribenzoate was reported in 1951 by Fischer and Haselbach, who found that the reagent slowly inactivated the enzyme from malted barley. As they found no sulphur in the amylase, and the loss of activity proceeded much more slowly than with β -amylase (an enzyme which is known to require sulphydryl groups for activity), they concluded that the reagent was deactivating the α -amylase by some mechanism other than by mercaptide formation.

Di Carlo and Redfern (1947) obtained results indicating that p-chloromercuribenzoate slowly inhibits the α -amylase of B. subtilis at pH 4.7, but has no effect at pH 6. But Akabori et al. (1956) and Junge et al. (1959) determined the amino-acid composition of this amylase, and found no cystine or cysteine residues. Therefore the mercuribenzoate must be capable of attacking the bacterial amylase at sites other than sulphydryl groups.

Other workers have shown that p-chloromercuribenzoate is not completely specific for such groups e.g. Sohler et al. (1952) found that catalase was inhibited by the reagent, although sulphydryl groups were not considered important in the enzyme. As yet, however, the mechanism of attack on non-sulphydryl enzymes is not understood.

It is possible, then, that in the broad-bean and cereal α -amylases sulphydryl groups are not important for enzymic activity; a similar conclusion has been reported for mammalian, fungal and bacterial α -amylases [Fischer and Stein (1960)].

Molecular size of the cereal α -amylases: Gel filtration may be used to determine the molecular weights of enzymes, if the gel has first been calibrated using proteins of known size.

The ratio
$$\frac{\text{elution volume of the enzyme } (V_e)}{\text{column void volume } (V_o)}$$
 was found to be the same for the α -amylases of wheat, malted wheat and malted barley on a column of Bio-Gel P-100 (Table 4.12).

This shows that the enzyme molecules are of similar sizes, and that no large change in the molecular weight of a cereal α -amylase takes place on germination.

From Fig. 4.17, the molecular weight was estimated to be ca. 50,000. However, this must be regarded as a minimum value, as it is possible for an enzyme to be retained more by a gel than would be expected for simple molecular sieving (e.g. by hydrogen-bonding), thus giving a falsely high ratio $\frac{V_e}{V_o}$, and hence a low molecular weight.

Schwimmer and Balls (1949) reported that the molecular weight of malted barley α -amylase is ca. 59,000.

Section 5

The Action-Pattern of the α -Amylases

5.

Introduction

The action-pattern of the α -amylases on the starch components has been the subject of much controversy. Meyer and Bernfeld (1941) proposed the theory [later restated by Meyer and Gonon (1951)] that, in the hydrolysis of amylose by any α -amylase, the enzyme is unable to attack the linkages at the ends of a substrate molecule, but all other bonds are hydrolysed at random.

The end-products of the reaction would then be maltose and maltotriose. However, these workers found glucose as a product of the action of pancreatic and malt α -amylases, and concluded that the monosaccharide was formed only by the slow hydrolysis of maltotriose. The final products from amylose were thus predicted to be glucose and maltose in the ratio 1:3.35. In the experiments of Meyer and Bernfeld, or Meyer and Gonon, complete conversion was not achieved, but the results from partial hydrolyses of amylose by malt and pancreatic enzymes seemed to confirm the theory.

Bernfeld (1951) recognised that the Kinetics of starch hydrolysis varied according to the source of the α -amylase used, but attributed this to differences in the affinities of the various enzymes for the substrates, and not to differences in the action-patterns.

Myrbäck (1950), on the other hand, considered that the action-patterns of plant and mammalian α -amylases were not identical. He concluded that malt α -amylase

required at least 6-8 adjacent α -1:4-linked glucose units in a substrate in order to exhibit maximum activity, but that the requirement was less for mammalian enzymes.

Differences in the affinities of malted barley and mammalian amylases for short oligosaccharides were demonstrated by Meyer and Gonon (1951) and Svanborg and Myrbäck (1953). The latter workers showed that salivary α -amylase attacked maltohexose and starch at comparable rates, while the malted barley enzyme hydrolysed the polysaccharide six times faster than the dextrin.

Svanborg and Myrbäck (1953) considered that glucose was liberated from starch, and not from maltotriose, by the plant amylase. This glucose was thought to be released from the reducing end of a polysaccharide chain for no glucose was found in a digest containing maltohexonic acid, although maltohexose was degraded to glucose and maltose.

Other workers [Hanes and Cattle (1938), Kung et al. (1953)] showed, by measurement of iodine-staining ability and reducing power of starch-amylase digests, that α -amylases from various sources did not all exhibit the same degradation pattern.

Bird and Hopkins (1954) studied the products of the action of malted barley, B. subtilis and human salivary α -amylase on amylose and amylopectin by paper chromatography. They found that the plant and bacterial amylases degraded amylose ultimately to glucose and maltose, and concluded

that the glucose was not produced wholly by hydrolysis of maltotriose. The final ratio of glucose to maltose in the B. subtilis enzyme digest was 5.45:1, while that in the malt enzyme digest would probably have been 4.5:1 (if the reaction had gone to completion). Neither of these values is close to that predicted by Meyer and Bernfeld (1941).

The main products, in the early stages of the action of the bacterial and plant enzymes on amylose, were oligosaccharides (DP = 6-8) and maltotriose or maltose, respectively; in the salivary amylase digest the main products were maltose and maltotriose.

Bird and Hopkins (1954) put forward the theory that the five linkages nearest the non-reducing chain-end of a substrate molecule, and one or two bonds nearest the reducing-end of a chain, were fairly resistant to attack by the malted barley and B. subtilis α -amylases, respectively. The enzymes could, however, readily attack the second or third bonds (for plant and bacterial enzymes, respectively) from the reducing end, and all other bonds would be equally susceptible to enzymic action. Further, they considered that salivary α -amylase could not easily attack the first two linkages from the non-reducing end of a chain, or the linkage at the reducing end, and that the enzyme readily split the second bond from the reducing end; all other bonds would be randomly hydrolysed.

This theory is in agreement with that of Myrbäck concerning the action of malted barley α -amylase, but contradicts that of Meyer and Bernfeld.

Pazur (1953) and Pazur and Budovich (1956) showed the theory of Meyer and Bernfeld to be incorrect for the amylase of human saliva. They found (using radioactive substrates) that not all non-terminal bonds of maltopentose and maltohexose were equally readily attacked by the enzyme, and that maltotetrose was hydrolysed to give some glucose and maltotriose, as well as maltose. Pazur (1953) and Pazur and Budovich (1955) demonstrated the hydrolysis of maltotriose by salivary α -amylase. Although Myrbäck and Leissner (1944) failed to find such an attack, Walker and Whelan (1960) showed that the hydrolysis proceeded at a measurable rate, only if very high concentrations of salivary α -amylase were used. Maltotriose is thought to be the smallest saccharide hydrolysed by α -amylases [Whelan (1960)].

Dube and Nordin (1962) and Robyt and French (1963) studied the action-patterns of α -amylases from sorghum and B. subtilis, respectively. Their results were comparable to those of Bird and Hopkins (1954) for plant and bacterial enzymes. In each case a predominance of dextrans, $DP \approx 6$, was found. For the bacterial enzyme, Robyt and French (1963) found a high proportion of maltotriose. The latter workers showed that the attack of the B. subtilis amylase on small oligosaccharides was

essentially non-random. They explained their findings in terms of a dual specificity for the formation of maltohexose and maltotriose.

Similar results were obtained by MacGregor (1964) for α -amylases from soya- and broad-beans, barley and malted barley i.e. large amounts of malto-hexose and -heptose were formed by each enzyme, and the action of the malted barley amylase on oligosaccharides was non-random. MacGregor showed, however, that the amylolysis of amylose was random in the initial stages.

In summary, the results of most workers indicate that the hydrolysis of amylose by α -amylases consists initially of random attack on internal linkages. The amylolysis of bonds near chain-ends is not random, and so, as the polysaccharide is degraded, and the proportion of chain-ends increases, the enzymic attack becomes less random. The position and rate of attack on small dextrans varies according to the source of the α -amylase. Bacterial and plant amylases are capable of liberating small amounts of glucose from long substrate molecules, while the mammalian enzymes produce glucose mainly by the slow hydrolysis of maltotriose.

The amylolysis of amylopectin is complicated by the presence of α -1:6-bonds in the polysaccharide. These linkages are not only resistant to attack by α -amylases, but are capable of conferring resistance on adjacent α -1:4 bonds. Thus the hydrolysis of amylopectin is

random, except for bonds near a substrate chain-end or branch point. The source of an α -amylase determines the number and position of α -1:4 bonds near a 1:6 bond which are resistant to that enzyme. The smallest branched sugar obtained from amylopectin by the action of salivary, porcine pancreatic and A. oryzae amylases was 6³- α -glucosylmaltotriose [nomenclature as in Whelan (1960 a)]; from malt barley α -amylase action the smallest dextrin was panose (6²- α -glucosylmaltose), and the enzyme of B. subtilis produced 6²- α -maltosylmaltotriose as the smallest resistant saccharide [Whelan (1960)].

This Section describes a study of the action-pattern of the α -amylases from oats, rye and wheat on amylose and linear maltodextrins. In addition, the actions of various plant, bacterial and mammalian α -amylases on amylose have been compared quantitatively. Yields of dextrins to be expected at various stages of the degradation have been calculated for different theories of α -amylase action and compared with the experimentally-determined values.

Experimental and Results

Linear amylose and maltodextrins were used as substrates, so that examination of the products was not complicated by the presence of branched saccharides.

5a. Kinetics of the initial hydrolysis of amylose by the cereal α -amylases

The kinetics of the initial stage of amylolysis were followed viscometrically at 25°C. The specific viscosity, η_{sp} , of a solution of amylose (30 ml.; 0.2%; DP \approx 3,000) in 0.02 M acetate buffer (pH 4.8) was determined. After the addition of oat α -amylase solution (0.075 ml.; 0.11 units), η_{sp} was measured at intervals for 18 hours.

The experiment was repeated using amylose solution (20 ml.; 0.2%; DP \approx 3,000) in 0.02 M acetate buffer (pH 5.5) and enzyme (0.3 ml.; 0.45 units). The specific viscosity of the mixture was determined at intervals for 3.5 hours.

Similar experiments were carried out with amylose solution (20 ml.; 0.2%; DP \approx 2,100) in 0.02 M acetate buffer (pH 5.5) and rye or wheat α -amylase solution (0.1 ml.; 0.2 units or 0.02 ml.; 0.4 units, respectively). The specific viscosity was measured over periods of 9 and 6.5 hours, respectively.

The inverse of the degree of polymerisation, ($\frac{1}{DP}$), of amylose of specific viscosity, η_{sp} , was calculated from the

FIG. 5.1

Action of oat α -amylase
on amylose

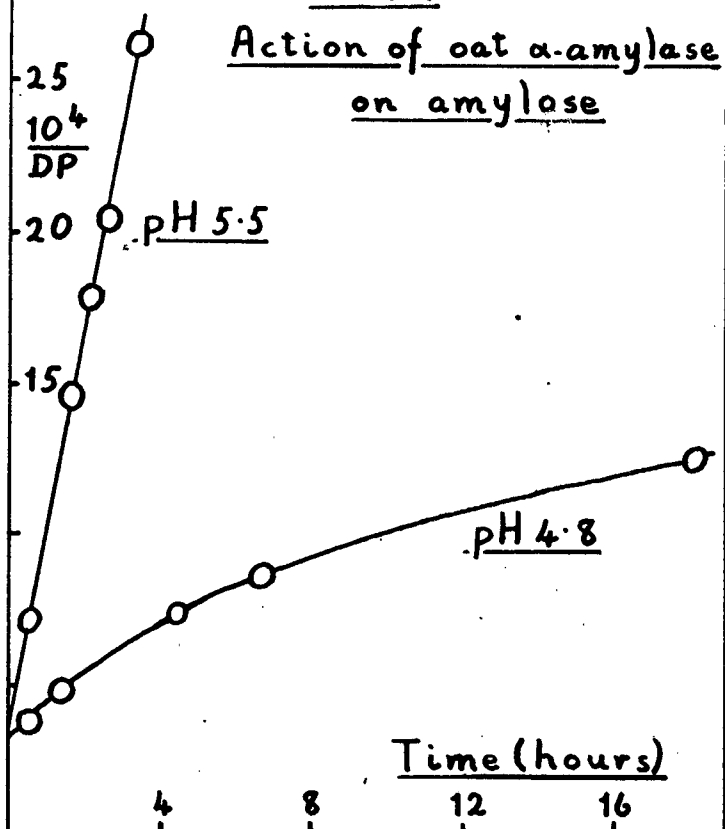


FIG. 5.2

Action of rye α -amylase
on amylose

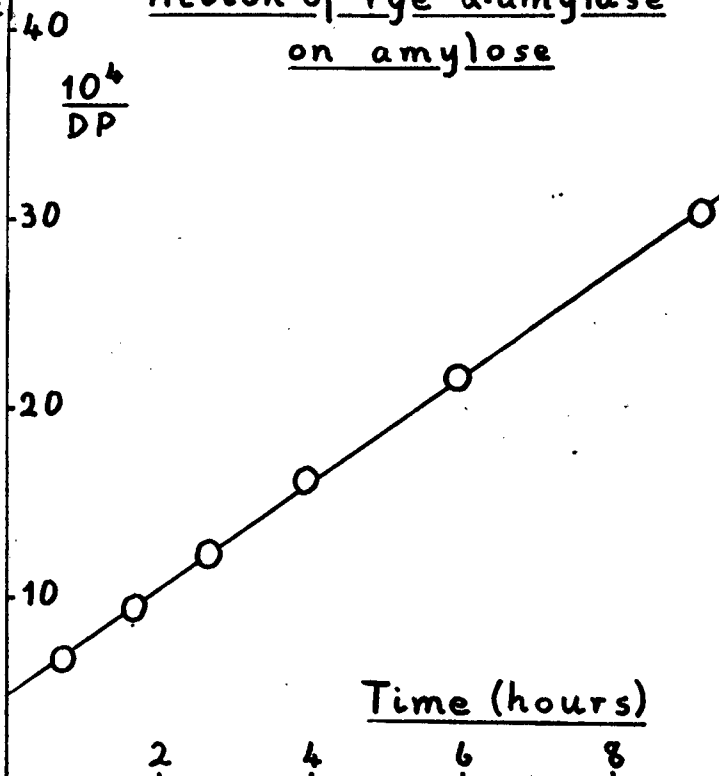


FIG. 5.3

Action of wheat α -amylase
on amylose

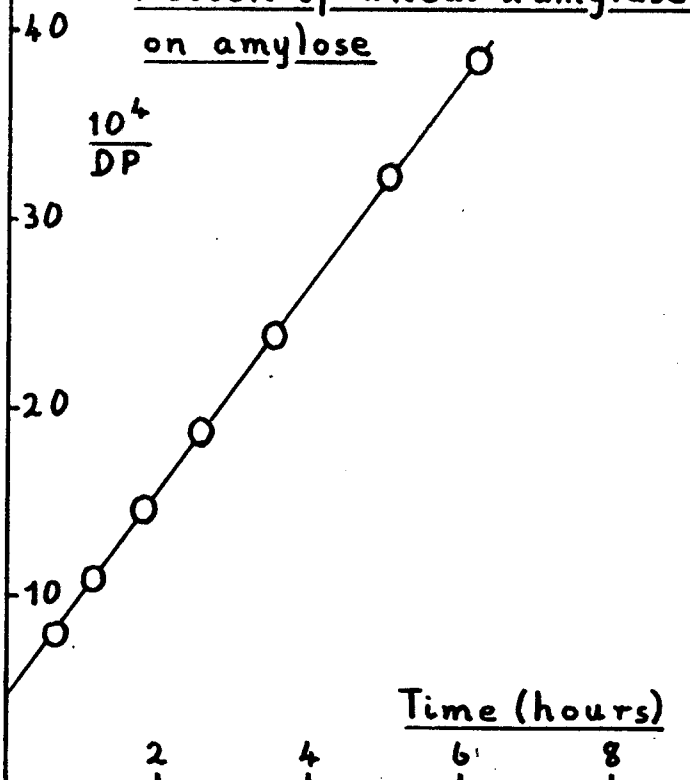
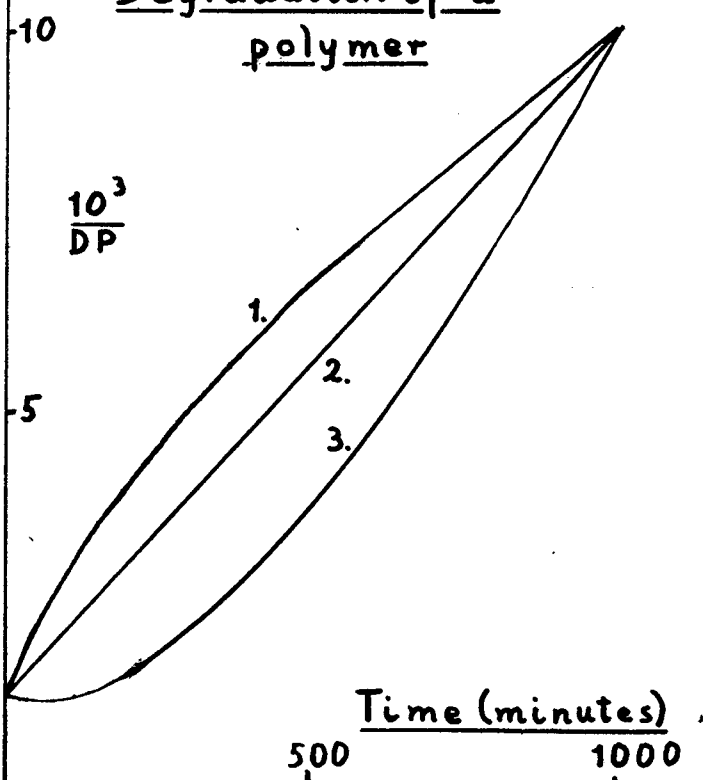


FIG. 5.4

Degradation of a
polymer



equation of Vink (1963):

$$\frac{1}{DP} = [K'c \left(\frac{1}{\eta_{sp}} + k \right)] \frac{1}{\alpha}$$

where c is the polymer concentration, K' and α are constants for the polymer-solvent system under consideration and k is Huggin's constant (See Discussion). Values of K' , η_{sp} and α were taken from the results of Banks and Greenwood (1963) for the amylose-water system.

Graphs were drawn of $\frac{1}{DP}$ versus time, and the results are shown in Figs. 5.1-5.3.

5b. Qualitative study of the production
of maltodextrins from amylose

Unbuffered amylose solution (7 ml.; 0.3%) and oat α -amylase solution (0.3 ml.; 0.6 units) were incubated at 35°C. Aliquots (5 ml.) were removed at intervals for examination by paper chromatography (see Section 2f.), and at the same time, samples (0.1 ml.) were stained with iodine (0.03 ml.; 0.2% iodine in 2% potassium iodide solution). After 150 hours, a further 0.3 ml. enzyme solution was added.

A second digest contained amylose solution (3 ml.; 0.3%) and oat enzyme (1.2 ml.; 2.4 units), and from this samples were removed and treated as above.

The results of these qualitative investigations are shown in Table 5.01.

Table 5.01

Action of oat α -amylase on amylose

Maltodextrin	Digest incubation time (hours)							
	Digest 1						Digest 2	
	21	45	70	141	165	189	444	72
G ₁	0	0	0	0	0	+	2+	3+
G ₂	+	2+	3+	4+	5+	6+	7+	8+
G ₃	+	2+	3+	4+	4+	5+	5+	6+
G ₄	+	2+	2+	3+	4+	4+	5+	5+
G ₅	+	2+	2+	3+	3+	4+	4+	5+
G ₆	+	2+	3+	4+	5+	6+	7+	8+
G ₇	+	2+	3+	4+	5+	6+	7+	7+
G ₈	+	2+	3+	4+	4+	4+	3+	2+
>G ₈	3+	4+	4+	4+	3+	2+	+	0
Iodine stain	purple-blue	purple	red-purple	red	orange	orange-yellow	achroic	achroic

G₁, G₂, G₃ ... etc. represent glucose, maltose, maltotriose ... etc.

The symbols +, 2+, etc. give an estimate of the relative amounts of each sugar on the chromatogram; quantities can be compared horizontally only.

A digest was set up containing unbuffered amylose solution (7 ml.; 0.3%) and rye α -amylase (2 ml.; 4 units) at 35°C. Aliquots were withdrawn for chromatographic examination and iodine-staining as before. After 71 hours, a further 3 ml. enzyme solution was added to the digest.

The results are given in Table 5.02.

Table 5.02

Action of rye α -amylase on amylose

Malto-dextrin	Digest incubation time (hours)						
	18	22	29	47	71	96	168
G ₁	0	0	+	+	2+	3+	3+
G ₂	+	2+	3+	4+	5+	6+	7+
G ₃	+	2+	2+	3+	4+	5+	5+
G ₄	+	2+	2+	3+	3+	4+	4+
G ₅	+	2+	2+	3+	3+	4+	4+
G ₆	+	2+	3+	4+	5+	6+	7+
G ₇	+	2+	3+	4+	5+	6+	6+
G ₈	+	2+	3+	4+	4+	3+	2+
G ₉	+	2+	3+	3+	2+	+	0
>G ₉	3+	4+	3+	2+	+	0	0
Iodine stain	purple red orange achroic achroic achroic achroic						

A digest was prepared from amylose solution (8 ml.; 0.3%) and wheat α -amylase solution (0.2 ml.; 8 units). Samples of this mixture were treated as above. Further aliquots of enzyme (0.2 ml.) were added after 48 and 73 hours. The results were as in Table 5.03.

Table 5.03

Action of wheat α -amylase on amylose

Maltodextrin	Digest incubation time (hours)							
	6	11	23	31	47	55	73	147
G ₁	0	0	+	2+	3+	4+	5+	6+
G ₂	+	2+	3+	4+	5+	6+	7+	8+
G ₃	+	+	2+	3+	4+	4+	5+	5+
G ₄	+	+	2+	2+	3+	3+	4+	4+
G ₅	+	+	2+	2+	3+	3+	3+	4+
G ₆	+	2+	3+	4+	5+	6+	7+	8+
G ₇	+	2+	3+	4+	5+	6+	7+	7+
G ₈	+	+	2+	3+	4+	4+	3+	2+
G ₉	+	+	2+	3+	2+	2+	+	0
>G ₉	3+	4+	5+	4+	3+	2+	+	0
Iodine stain	purple	red	red- orange	orange- yellow	achroic	achroic	achroic	achroic

5c.

Qualitative study of the action of
plant α -amylases on maltodextrins

Digests were prepared containing oligosaccharide solution (0.8 ml.; 0.2% of G_4 and G_5 or 0.5% of G_6 , G_7 or G_8) and α -amylase solution (0.2 ml.; 2 units bean enzyme or 5 units ungerminated cereal enzyme, or 200 units malted wheat enzyme). The mixtures containing the bean and malted wheat enzymes were left at 18°C under toluene, while the remainder were incubated at 35°C under toluene. Aliquots (0.15 ml.) were removed at intervals for chromatographic analysis.

The results are shown in Tables 5.04-5.06.

Table 5.04

Products of the action of soya- and broad-bean
 α -amylases on oligosaccharides

Substrate	Digest incubation time (hours)			
	19	43	90	114
G_4	0	G_2 +	G_2 2+	G_2 3+
G_5	0	G_2, G_3 +	G_2, G_3 2+	G_2, G_3 2+
G_6	$G_3; G_2, G_4$ + +	$G_3; G_2, G_4$ 2+ 2+	$G_3; G_2, G_4$ 3+ 3+	$G_3; G_2, G_4$ 4+ 4+
		G_1, G_5 +	G_1, G_5 2+	G_1, G_5 2+
G_7	$G_1, G_6; G_3, G_4$ + +	$G_1, G_6; G_3, G_4$ 2+ 2+	$G_1, G_6; G_3, G_4$ 3+ 3+	$G_1, G_6; G_3, G_4$ 4+ 3+
	G_2, G_5 +	G_2, G_5 +	G_2, G_5 2+	G_2, G_5 2+

Table 5.05

Products of the action of oat, rye and wheat α -amylases on oligosaccharides

Substrate	Digest incubation time (hours)		
	25	48	95
G ₄	G ₂ ; G ₁ ,G ₃ 2+ +	G ₂ ; G ₁ ,G ₃ 3+ +	G ₂ ; G ₁ ,G ₃ 4+ 2+
G ₅	G ₂ ,G ₃ +	G ₂ ,G ₃ ; G ₁ ,G ₄ 2+ +	G ₂ ,G ₃ ; G ₁ ,G ₄ 3+ 2+
G ₆	G ₂ ,G ₄ ; G ₃ + +	G ₂ ,G ₄ ; G ₃ 2+ +	G ₂ ,G ₄ ; G ₃ ; G ₁ ,G ₅ 3+ 2+ +
G ₇	G ₃ ,G ₄ ; G ₁ ,G ₆ ; G ₂ ,G ₅ + + +	G ₃ ,G ₄ ; G ₁ ,G ₆ ; G ₂ ,G ₅ 2+ 2+ +	G ₃ ,G ₄ ; G ₁ ,G ₆ ; G ₂ ,G ₅ 3+ 3+ +
G ₈	G ₂ ,G ₆ ; G ₁ ,G ₇ ; G ₄ 2+ + +	G ₂ ,G ₆ ; G ₁ ,G ₇ ; G ₄ 3+ 2+ + G ₃ ,G ₅ +	G ₂ ,G ₆ ; G ₁ ,G ₇ ; G ₄ 4+ 2+ 2+ G ₃ ,G ₅ +

Table 5.06

Products of the action of malted wheat
 α -amylase on oligosaccharides

Substrate	Digest incubation time (hours)
	67
G ₄	G ₂ ; G ₁ ,G ₃ 2+ +
G ₅	G ₂ ,G ₃ ; G ₁ ,G ₄ 2+ +
G ₆	G ₂ ,G ₄ ; G ₃ ; G ₁ ,G ₅ 3+ 2+ +
G ₇	G ₁ ,G ₆ ; G ₃ ,G ₄ ; G ₂ ,G ₅ 3+ 2+ +
G ₈	G ₂ ,G ₆ ; G ₁ ,G ₇ ; G ₄ ; G ₃ ,G ₅ 4+ 3+ 2+ +

5d. Quantitative study of the action
of α -amylases on amylose

In each case, unbuffered amylose solution (0.3%) was incubated with enzyme at 35°C, and aliquots (10 ml.) were removed at intervals after the achroic limit. The samples were boiled for 5 minutes and evaporated to dryness on a rotary evaporator; the sugars were then dissolved in distilled water (1.5 ml.) and applied to Whatman 3MM chromatography paper. After separation by ascending chromatography (see Section 2f.),

the oligosaccharides were removed from the paper with distilled water, and estimated by the alkaline ferricyanide method (See Section 2d.).

(i) Soya-bean α -amylase: Amylose solution (10 ml.) and enzyme solution (4 units) were incubated at 35°C. The digest was achroic after 72 hours and was evaporated after 120 hours.

A second digest contained amylose solution (20 ml.), p-chloromercuribenzoic acid (3 mg.) and enzyme solution (50 units). After 60 hours, a further 25 units of α -amylase were added to the mixture. The results are shown in Table 5.07.

Table 5.07

Action of soya-bean α -amylase on amylose

Digest incubation time (hours)	% by weight of						
	G ₁	G ₂	G ₃	G ₄	G ₅	G ₆	>G ₆
120 (digest 1)	4	13	9	7	5	21	41
60 (digest 2)	3	9	10	6	5	17	50
120 (digest 2)	7	13	11	7	6	31	25

The figures have been quoted to the nearest whole number.

(ii) Broad-bean α -amylase: A digest was prepared containing amylose solution (30 ml.) and enzyme solution (100 units). After 58 hours, two samples were removed for analysis, and 30 units of enzyme were added to the

remainder of the digest. The composition of the oligosaccharide mixtures were as below:

Table 5.08

Action of broad-bean α -amylase on amylose

Digest incubation time (hours)	% by weight of						
	G ₁	G ₂	G ₃	G ₄	G ₅	G ₆	>G ₆
58	2	8	10	6	6	14	54
	2	8	9	6	6	14	55
104	5	13	11	7	7	28	29

(iii) Oat α -amylase: The digest contained amylose (20 ml.) and enzyme (12 units) and became achroic after 44 hours. The results were as given in Table 5.09.

Table 5.09

Action of oat α -amylase on amylose

Digest incubation time (hours)	% by weight of						
	G ₁	G ₂	G ₃	G ₄	G ₅	G ₆	>G ₆
49	1	10	5	6	5	17	57
92	1	13	6	7	6	22	46

(iv) Rye α -amylase: Amylose (30 ml.) was incubated with enzyme (30 units); further additions of enzyme (60 units, 40 units and 60 units) were made after 23, 45 and 86 hours, respectively. The results are shown in Table 5.10.

Table 5.10Action of rye α -amylase on amylose

Digest incubation time (hours)	% by weight of						
	G ₁	G ₂	G ₃	G ₄	G ₅	G ₆	>G ₆
45	1	9	4	5	5	14	62
86	1	15	5	6	7	26	40
130	2	18	5	7	8	30	30

(v) Wheat α -amylase: To a digest containing 20 ml. amylose were added three aliquots of enzyme solution, each containing 24 units of enzyme, at intervals of 34 hours. The results are given in Table 5.11.

Table 5.11Action of wheat α -amylase on amylose

Digest incubation time (hours)	% by weight of						
	G ₁	G ₂	G ₃	G ₄	G ₅	G ₆	>G ₆
72	1	7	3	5	5	12	67
95	1	9	4	6	6	14	60

(vi) Malted barley α -amylase: A digest was prepared containing amylose (20 ml.) and enzyme solution (60 units). Further aliquots of enzyme (60, 30 and 30 units) were added after 8, 27 and 51 hours, respectively. The results were as in Table 5.12.

Table 5.12Action of malted barley α -amylase on amylose

Digest incubation time (hours)	% by weight of						
	G ₁	G ₂	G ₃	G ₄	G ₅	G ₆	>G ₆
37	1	7	4	4	5	14	65
72	1	10	5	6	6	19	53

(vii) Malted wheat α -amylase: The digest consisted of amylose (30 ml.) and enzyme (300 units). After 26 hours, 100 units of enzyme were added to the mixture.

A second digest contained 10 ml. amylose solution and 1,000 units of enzyme. The compositions of the oligosaccharide mixtures from the digests are shown below.

Table 5.13Action of malted wheat α -amylase on amylose

Digest incubation time (hours)	% by weight of						
	G ₁	G ₂	G ₃	G ₄	G ₅	G ₆	>G ₆
6 (digest 1)	1	9	4	5	5	16	60
22 (digest 1)	2	15	5	8	8	35	27
48 (digest 1)	4	15	5	8	9	39	20
1.4 (digest 2)	1	8	4	6	6	18	57

(viii) Porcine pancreatic α -amylase: Two digests were prepared, the first containing amylose (20 ml.) and enzyme (700 units), the second 10 ml. amylose and 50 units

of α -amylase. The first digest was achroic after 23 minutes. Table 5.14 shows the results obtained.

Table 5.14

Action of porcine pancreatic α -amylase on amylose

Digest incubation time (hours)	% by weight of						
	G ₁	G ₂	G ₃	G ₄	G ₅	G ₆	>G ₆
0.4 (digest 1)	1	42	25	15	3	2	12
1.8 (digest 1)	2	52	36	10	-	-	-
17 (digest 2)	1	44	31	21	1	1	1

(ix) Bacillus subtilis α -amylase: A digest was set up, containing amylose solution (20 ml.) and enzyme solution (3,000 units). The results are given in Table 5.15.

Table 5.15

Action of Bacillus subtilis α -amylase on amylose

Digest incubation time (hours)	% by weight of						
	G ₁	G ₂	G ₃	G ₄	G ₅	G ₆	>G ₆
0.5	1	9	24	9	10	28	19
1.3	2	11	24	9	14	30	10

Discussion

Kinetics of the initial hydrolysis of amylose by cereal α -amylases: The main problem was to determine whether the initial degradation of the polysaccharide was occurring by a random, or a non-random, process. Ideally, the depolymerisation should be studied by number-average methods, counting the number of molecules in the system. However, such methods are insensitive to limited degrees of degradation, and in this situation weight-average techniques must be used; of these, viscometry is the most sensitive to the initial scission of bonds in a polymer.

The degree of polymerisation (DP) of the polymer may be calculated from viscosity measurements, using the Mark-Houwink equation $[\eta] = K'(\text{DP})^\alpha$, where $[\eta]$ is the limiting viscosity number, and K' and α are constants for the polymer-solvent system.

Bryce and Greenwood (1957) pointed out that the rate of a first-order reaction is proportional to $\frac{1}{\text{DP}}$. As it was more convenient to follow the α -amylolyses by changes in the specific viscosity of the substrate (η_{sp}) with time, the equation of Vink (1963) was used:

$$\frac{1}{\text{DP}} = [K'c(\frac{1}{\eta_{\text{sp}}} + k)]^{\frac{1}{\alpha}} \quad 1.$$

k is Huggin's constant and was taken as 0.65 [Everett and Foster (1959)]; α was taken as 0.5 [Banks and Greenwood (1963)].

Vink showed that, for random degradation of a polymer, the change of DP with time (t) is given by

$$\frac{1}{\overline{DP}} = \frac{1}{(\overline{DP})_0} + k't . \quad 2.$$

if the original chain-length distribution of the polymer was random. $(\overline{DP})_0$ is the initial degree of polymerisation and k' is a velocity constant. Thus a graph of $\frac{1}{\overline{DP}}$ versus t should be a straight line for random hydrolysis (Fig. 5.4, curve 2).

Vink demonstrated further that other types of degradation process produce graphs of different shapes. During hydrolysis of a polymer containing weak links, a rapid scission of these bonds would occur and then the more resistant linkages would be broken at a slower rate; for the graph of $\frac{1}{\overline{DP}}$ versus t , a curve such as Fig. 5.4, curve 2 would result. On the other hand, a graph such as Fig. 5.4, curve 3 would be obtained if depolymerisation proceeded by a "zip" mechanism i.e. if small units were successively split off from the polymer chain-ends. Vink emphasised that failure to obtain a straight-line graph did not necessarily mean that degradation was non-random. Changes in the rate constant k' could alter the kinetics of depolymerisation, while the over-all process could still occur in a random manner.

A study of the hydrolysis of amylose by oat, rye and wheat α -amylases at pH 5.5 yielded straight lines for the graphs of $\frac{1}{\overline{DP}}$ versus t [Figs. 5.1 (pH 5.5), 5.2 and 5.3]. This indicates that, at least in the initial stages, α -amylolysis involves the random scission of the α -1:4-links of amylose molecules. Similar results were obtained by

MacGregor (1964) for the α -amylases of soya- and broad-beans, barley and malted barley, and also for the acid hydrolysis of amylose - a known random process.

At pH 4.8, however, the action of the oat amylase was such that the graph of $\frac{1}{DP}$ versus t was curved [Fig. 5.1 (pH 4.8)]. This was most probably caused, not by a change in action-pattern, but by the instability of the enzyme at the lower pH. It was found that oat α -amylase lost 5% of its activity on standing at 18°C for 65 minutes at pH 4.8 [Fig. 4.11], and hence the rate of the enzyme reaction would be expected to decrease with time, in the viscometer at 25°C.

It must be emphasised that these results represent only the initial stage of the degradation of amylose. Although a large change took place in the degree of polymerisation of the polysaccharide molecules (ca. 7-fold), a very small fraction of the original bonds had been broken.

The number of bonds broken/molecule (p) during a random hydrolysis may be calculated from the equation

$$p = \frac{(DP)_0}{DP} - 1 \quad [\text{Cowie and Greenwood (1957)}]$$

Thus for the α -amylolyses represented in Figs. 5.1-5.3, the number of bonds broken/molecule ≈ 6 . As the DP of the amylose used was ca. 3,000 (for the hydrolysis by oat α -amylase), or ca. 2,100 (for the rye and wheat amylases), the percentages of bonds broken during the degradations were 0.2 and 0.3, respectively.

Qualitative study of the production of maltodextrins from amylose: Tables 5.01-5.03 show the maltodextrins present at various stages of the hydrolysis of amylose by oat, rye and wheat α -amylases. In each case, the initial sample was taken when the amylolysis had proceeded further than for the viscosity measurements.

The results indicate that the action-patterns of the three amylases are similar. Initially, maltodextrins larger than G_8 predominated in the digests, but traces of smaller sugars G_2 - G_8 were also present. These short dextrins were probably formed directly from amylose molecules and not from oligosaccharides. As the hydrolysis continued, all the saccharides detected on the chromatograms (G_2 - G_{11}) increased in amount, and then the higher ones (G_8 - G_{11}) began to decrease. At the achroic limit, no sugar greater than G_{10} was present in the digests.

The rate of hydrolysis of these oligosaccharides was extremely slow compared to that of amylose; further additions of enzyme were therefore made during the hydrolyses to counteract the slowing-down of the reaction. Another factor contributing to the decrease of the degradation rate was the instability of the enzymes during prolonged incubations at 35°C.

The oat, rye and wheat amylases seem to resemble malted barley α -amylase in that the rate of attack on oligosaccharides is very much lower than that on polysaccharides [Schwimmer (1950), Meyer and Gonon (1951), Svanborg and Myrbäck (1953)].

Glucose appeared relatively late in the digests, at the stage where the iodine-stain was orange, and then increased as the hydrolysis proceeded. Meyer and Bernfeld (1941) and Meyer and Gonon (1951) considered that glucose was formed only by the hydrolysis of maltotriose, while Svanborg and Myrbäck (1953) concluded that glucose could be produced from large as well as small molecules. Bird and Hopkins (1954) also thought that malt α -amylase was capable of splitting glucose from long chains.

As the action of the oat, rye and wheat α -amylases on small oligosaccharides was extremely slow, it seems unlikely that the enzymes could hydrolyse sufficient maltotriose before the achroic limit to account for the glucose produced. It is probable, therefore, that these amylases can attack - although with difficulty - a terminal bond of oligosaccharides larger than maltotriose to yield glucose.

The main products present in the last samples from each digest were G_2 , G_6 and G_7 with smaller amounts of G_1 , G_3 , G_4 , G_5 and G_8 . The results are very similar to those obtained by Bird and Hopkins (1954) and Dube and Nordin (1962) for the α -amylases of malted barley and sorghum, respectively.

Qualitative study of the action of some plant α -amylases on maltodextrins: The results in Tables 5.04-5.06 indicate that the main products of attack on short oligosaccharides were similar for soya-bean, broad-bean, oat, rye, wheat, and malted wheat α -amylases, and that the action was non-random.

In all cases, G_4 was hydrolysed extremely slowly and yielded predominantly G_2 , but the cereal amylases also

produced a little G_1 and G_3 . Pazur (1953) showed that salivary α -amylase attacked G_4 to give mostly G_2 , and some G_1 and G_3 .

The fission products most readily formed from G_5 were G_2 and G_3 ; G_1 and G_4 were found in smaller amounts in the cereal enzyme digests. As the hydrolysis of short oligosaccharides was extremely slow, it is possible that G_1 and G_3 or G_4 might have been produced by prolonged incubation of the bean amylases with G_4 or G_5 , respectively.

G_6 was degraded to give G_2 , G_3 and G_4 preferentially, with much less G_1 and G_5 . Bird and Hopkins (1954) obtained similar results for malted barley α -amylase. From G_7 , the main products were G_1 and G_6 , G_3 and G_4 , while traces of G_2 and G_5 were present. The action of the cereal enzymes on G_8 yielded mostly G_2 and G_6 , less G_1 , G_4 and G_7 and very small amounts of G_3 and G_5 . Greenwood and MacGregor (1965) observed comparable products from the action of malted barley α -amylase.

However, the α -amylase of *B. subtilis* was shown by Robyt and French (1963) to act rather differently on short oligosaccharides. The predominant products from G_6 were G_1 and G_5 ; from G_7 were G_1 and G_6 , G_2 and G_5 ; and from G_8 were $G_2 + G_6$, $G_3 + G_5$.

As glucose was formed directly from G_4 - G_8 by the action of the cereal amylases, and from G_6 - G_7 by the bean amylases, it seems very unlikely that the suggestion of Meyer and Bernfeld (1941), concerning the formation of glucose from maltotriose only, is correct.

For each enzyme studied, the rates of hydrolysis of the maltodextrins decreased in the order $G_8 > G_7 > G_6 > G_5 > G_4$.

Noticeable features of the results are that (i) glucose was not a major product, except from G_7 , (ii) G_5 was released only in very small quantities from the higher oligosaccharides, and (iii) G_2 was a major product, except from G_7 . It seems likely, then, that the first and fifth bonds from an oligosaccharide chain-end are fairly resistant to attack by plant α -amylases, while the second linkage from an end is readily cut. From the above results, it is not possible to decide whether the chain ends involved consist of reducing or non-reducing groups. Considering the results of other workers [e.g. Svanborg and Myrbäck (1953), Bird and Hopkins (1954), Pazur and Budovich (1956), Robyt and French (1963)] who used modified or radioactive substrates, it is probable that the resistant linkages are those at both ends of a chain (the bond at the reducing end being less resistant than that at the non-reducing end) and the fifth from the non-reducing end; the susceptible link is likely to be the bond second from the reducing end.

Quantitative study of the action of various α -amylases on amylose: Tables 5.07-5.15 show the proportions of maltodextrins present after the achroic limit of the hydrolysis of amylose by α -amylases of plant, bacterial and mammalian origins. Estimations were reproducible to ca. 7%. The instability of the α -amylases over long periods at 35°C necessitated addition of enzyme to some

of the digests as hydrolysis proceeded. The second soya-bean enzyme digest contained p-chloromercuribenzoate to ensure the inhibition of any β -amylase molecules which might have been undetected in previous tests. It was not possible to carry out the amylolyses by the cereal enzymes in the presence of mercuribenzoate, as the reagent inhibited cereal α - as well as β -amylases (Table 4.11).

A comparison of Tables 5.07 and 5.08 shows that the distributions of oligosaccharides produced by the bean α -amylases were very similar, but were different from those given by the other enzymes studied [Tables 5.09-5.15]. For the bean amylases, the main products were G_2 , G_3 and G_6 .

The results in Tables 5.09-5.13 indicate that all the cereal α -amylases have similar action-patterns, and that germination of a cereal causes no significant change in the mode of action of the α -amylase. The cereal enzymes produced less G_1 and G_3 but more G_2 than the bean amylases; hence the major products of cereal α -amylase action on amylose were G_2 and G_6 . This is in agreement with the qualitative results obtained (p. 111).

As the proportions of dextrans in the malted wheat digests 1 and 2 were comparable (Table 5.13, lines 1 and 4), it is probable that, during the hydrolysis of amylose, a 10-fold change in enzyme concentration has little effect on the distribution of products formed by cereal α -amylases, at least up to and just beyond the achroic limit. Kung et al. (1953) showed that the shapes of the curves of

iodine-staining ability versus reducing power of amylose, during hydrolysis by porcine pancreatic and A. oryzae α -amylases, did not vary with a 4-fold change of enzyme concentration.

If the actions of α -amylases from different sources are to be compared, however, experiments must be performed at the same concentration of substrate; Somogyi (1940) and Bird and Hopkins (1954) showed that the composition of the products for any one α -amylase can be varied by changing the initial substrate concentration.

The action-pattern of the porcine pancreatic amylase was quite different from those of the plant and bacterial enzymes studied. (Compare Table 5.14 with Tables 5.07-5.13, 5.15.) At the achroic point of the amylose-hydrolyses by cereal α -amylases ca. 65% of the saccharides were larger than G_6 (Tables 5.11 and 5.12), while at the corresponding stage in the pancreatic enzyme digest this figure was only 12%. The reducing power of the latter digest would be much higher than that of the former.

Hanes and Cattle (1938) found that, at the achroic limit, the reducing power of a solution containing amylose and porcine pancreatic amylase was almost twice as high as that of a similar digest with malted barley α -amylase. They proposed that the malt enzyme had a very much lower affinity for small dextrans than the mammalian amylase. Similar conclusions were reached by Meyer and Gonon (1951). Thus the malted barley enzyme would attack long glucosidic

chains preferentially, rendering an amylose solution achroic with a relatively small increase in the reducing power; the pancreatic amylase, attacking long and short dextrans with equal ease, would cause a large increase in reducing power before the achroic limit was reached.

There was no increase in the amount of G_6 in the pancreatic amylase digest after the achroic limit (Table 5.14), unlike the other α -amylases. This would be expected if the rate of attack of the enzyme on G_6 was fairly fast, so that the sugar would be degraded as soon as it was formed from higher oligosaccharides.

The main products, then, from the hydrolysis of amylose by the mammalian enzyme were G_2 , G_3 and G_4 ; very little G_1 was formed.

B. subtilis α -amylase exhibited yet another type of action-pattern (Table 5.15), more like the plant than the mammalian enzymes. The predominant sugars produced were G_3 and G_6 . These results are very similar to those obtained by Robyt and French (1963).

Theories of α -amylase action:

(i) Random degradation.

Kuhn (1930) showed that the weight-fraction (Y_n) of an oligomer of length n units (an n -mer), formed during random degradation of a polymer of length N , is given by

$$Y_n = ns^2(1-s)^{n-1},$$

3.

if $N \gg n$, and s is the degree of scission.

Yields of oligosaccharides were calculated from equation 3., values of s being chosen so that the total yield of oligomers $>G_6$ approximated to that found experimentally. For the pancreatic amylase (Table 5.14, lines 2 and 3) approximate values of s were calculated from the experimental proportions of the products. The degree of scission could not be calculated in this way for the other digests, as the distribution of sugars $>G_6$ was not known. As all the cereal α -amylases appeared to have a similar action-pattern (Tables 5.09-5.13), theoretical values have been calculated for comparison with the results of degradation by one cereal enzyme only, that of malted wheat (Table 5.13, lines 1-3).

Calculated yields are compared with experimental values in Tables 5.16-5.20 [assumption (1)].

Table 5.16
Calculated yields of maltodextrins;
soya-bean α -amylase action

Theoretical assumption	% by weight of						
	G ₁	G ₂	G ₃	G ₄	G ₅	G ₆	>G ₆
Experimental	4	13	9	7	5	21	41
(i) Random degradation	7.6	11.0	11.9	11.6	10.5	9.1	38.3
(11a) G ₅ stable, x = 0.38	2.5	5.1	7.6	10.1	12.7	--62.0--	
(11b) G ₆ stable, x = 0.59	2.8	5.6	8.4	11.2	14.0	16.9	41.0
Experimental	3	9	10	6	5	17	50
(i) Random degradation	5.1	7.8	9.1	9.4	9.1	8.5	^{51.0} (49.0)
(11a) G ₅ stable, x = 0.33	2.2	4.4	6.6	8.8	11.0	--67.0--	
(11b) G ₆ stable, x = 0.50	2.4	4.8	7.1	9.5	11.9	14.3	50.0
Experimental	7	13	11	7	6	31	25
(i) Random degradation	11.1	14.8	14.8	13.2	11.0	8.8	26.3
(11a) G ₅ stable, x = 0.44	2.9	5.9	8.8	11.7	14.7	--56.0--	
(11b) G ₆ stable, x = 0.75	3.6	7.1	10.7	14.3	17.9	21.4	25.0
(11i) See theory (11id), p. 127.	3.6	11.6	11.2	6.0	6.2	36.4	25.0

Table 5.17

Calculated yields of maltodextrins;
broad-bean α -amylase action

Theoretical assumption	% by weight of						
	G ₁	G ₂	G ₃	G ₄	G ₅	G ₆	>G ₆
Experimental	2	8	9	6	6	14	55
(1) Random degradation	4.0	6.4	7.7	8.2	8.2	7.9	57.6
(11a) G ₅ stable, x = 0.31	2.1	4.1	6.2	8.3	10.3	--69.0--	
(11b) G ₆ stable, x = 0.45	2.1	4.3	6.4	8.6	10.7	12.9	55.0
Experimental	5	13	11	7	7	28	29
(1) Random degradation	11.1	14.8	14.8	13.2	11.0	8.8	26.3
(11a) G ₅ stable, x = 0.43	2.9	5.7	8.6	11.5	14.3	--57.0--	
(11b) G ₆ stable, x = 0.71	3.4	6.8	10.1	13.5	16.9	20.3	29.0
(111) See theory (111d), p. 127.	3.1	11.6	11.2	6.0	6.2	32.9	29.0

Table 5.18Calculated yields of maltodextrins;malted wheat α -amylase action

Theoretical assumption	% by weight of						
	G ₁	G ₂	G ₃	G ₄	G ₅	G ₆	>G ₆
Experimental	1	9	4	5	5	16	60
(i) Random degradation	4.0	6.4	7.7	8.2	8.2	7.9	57.6
(iia) G ₅ stable, x = 0.24	1.6	3.2	4.8	6.4	8.0	--76.0--	
(iib) G ₆ stable, x = 0.40	1.9	3.8	5.7	7.6	9.5	11.5	60.0
Experimental	2	15	5	8	8	35	27
(i) Random degradation	11.1	14.8	14.8	13.2	11.0	8.8	26.3
(iia) G ₅ stable, x = 0.38	2.5	5.1	7.6	10.1	12.7	--62.0--	
(iib) G ₆ stable, x = 0.73	3.5	6.9	10.4	13.9	17.4	20.9	27.0
(iii) See theory (iiic), p. 127.	2.0	13.7	6.7	6.7	6.8	37.1	27.0
Experimental	4	15	5	8	9	39	20
(i) Random degradation	14.4	17.9	16.6	13.8	10.7	7.9	18.7
(iia) G ₅ stable, x = 0.41	2.7	5.5	8.2	10.9	13.7	--59.0--	
(iib) G ₆ stable, x = 0.80	3.8	7.6	11.4	15.2	19.1	22.9	20.0
(iii) See theory (iiic), p. 127.	3.0	13.7	6.7	6.7	6.8	43.1	20.0

Table 5.19

Calculated yields of maltodextrins;
porcine pancreatic α -amylase action

Theoretical assumption	% by weight of						
	G ₁	G ₂	G ₃	G ₄	G ₅	G ₆	>G ₆
Experimental	1	42	25	15	3	2	12
(1) Random degradation	19.4	21.8	18.3	13.7	9.6	6.4	10.8
(11a) G ₃ stable, x = 0.68	11.3	22.7	34.0	-----	32.0	-----	-----
(11b) G ₄ stable, x = 0.83	8.3	16.6	24.9	33.2	-----	17.0	-----
Experimental	2	52	36	10	0	0	0
(1) Random degradation	18.1	20.8	17.9	13.8	9.8	6.8	12.8
(11a) G ₃ stable, x = 0.90	15.0	30.0	45.0	-----	10.0	-----	-----
(11b) G ₄ stable, x = 1.00	10.0	20.0	30.0	40.0	0	0	0
(111) See theory (111e), p. 128.	0	52.5	37.5	10.0	0	0	0
Experimental	1	44	31	21	1	1	1
(1) Random degradation	15.4	18.7	17.1	13.9	10.6	7.7	16.6
(11a) G ₃ stable, x = 0.76	12.7	25.3	38.0	-----	24.0	-----	-----
(11b) G ₄ stable, x = 0.97	9.7	19.4	29.1	38.8	-----	3.0	-----
(111) See theory (111e), p. 128.	0	41.5	37.5	21.0	0	0	0

Table 5.20Calculated yields of maltodextrins;B. subtilis α -amylase action

Theoretical assumption	% by weight of						
	G ₁	G ₂	G ₃	G ₄	G ₅	G ₆	>G ₆
Experimental	1	9	24	9	10	28	19
(1) Random degradation	14.4	17.9	16.6	13.8	10.7	7.9	18.7
(11a) G ₅ stable, x = 0.53	3.5	7.1	10.6	14.2	17.7	---47.0---	
(11b) G ₆ stable, x = 0.81	3.9	7.7	11.6	15.4	19.3	23.1	19.0
(iii) See theory (iiif), p. 129.	3.1	7.6	19.4	5.4	5.9	39.8	19.0
Experimental	2	11	24	9	14	30	10
(1) Random degradation	20.3	22.4	18.4	13.5	9.3	6.2	9.9
(11a) G ₅ stable, x = 0.60	4.0	8.0	12.0	16.0	20.0	---40.0---	
(11b) G ₆ stable, x = 0.90	4.3	8.6	12.9	17.1	21.4	25.7	10.0
(iii) See theory (iiif), p. 129.	4.4	7.6	19.4	5.4	5.9	47.5	10.0

It can be seen (Tables 5.16-5.20) that the experimental values are quite different from the theoretical ones based on assumption (1) and so, notwithstanding the evidence of the viscosity experiments (p. 108), the hydrolysis of amylose cannot be random at the later stages. This is confirmed by the qualitative results from the amylolysis of maltodextrins (Tables 5.04-5.06).

For the bean enzymes, much less G_1 , G_4 and G_5 and more G_6 was formed than the theory of random degradation predicted; the cereal amylases produced less G_1 , G_3 , G_4 and G_5 and an excess of G_6 ; in the mammalian enzyme digest there was a deficiency of G_1 , G_5 and G_6 and an excess of G_2 and G_3 ; while the bacterial α -amylase yielded less G_1 , G_2 and G_4 and more G_3 and G_6 than predicted.

(ii) Random degradation to give products resistant to further attack.

The results of the experiments on the hydrolysis of maltodextrins by the plant α -amylases (p. 111) suggested that small oligosaccharides, i.e. G_6 and $<G_6$, were degraded much more slowly than larger sugars. Similar observations were reported by Robyt and French (1963) for the α -amylase of B. subtilis. Thus these small saccharides (G_6 and $<G_6$) may be regarded as effectively resistant to amylolysis if higher sugars are present in the digests.

Yields of maltodextrins were calculated, by the theory given below, for the random hydrolysis of amylose, assuming

that a) G_5 and $\langle G_5 \rangle$ were resistant to further attack, or
b) G_6 and $\langle G_6 \rangle$ were resistant.

For random degradation of a polymer of length N , the weight-fraction (Y_n) of n -mer present at degree of scission s may be calculated from the equation of Kuhn (equation 3., above), only if $N \gg n$. However, Montroll and Simha (1940) developed a more general equation, i.e.

$$Y_n = \frac{n}{N} [2s(1-s)^{n-1} + (N-n-1)s^2(1-s)^{n-1}] \quad 4.$$

Now the change in the yield with s is given by the

$$\begin{aligned} \text{differential } \frac{dY_n}{ds} = \frac{n}{N} [2(1-s)^{n-1} - 2s(n-1)(1-s)^{n-2} \\ + 2s(N-n-1)(1-s)^{n-1} - s^2(N-n-1)(n-1)(1-s)^{n-2}] \end{aligned} \quad 5.$$

The positive terms represent the formation of n -mer from larger oligomers, while the negative terms represent its degradation.

Painter (1963) pointed out that, if the n -mer is resistant to further attack, but all larger oligomers may be degraded, the final yield of n -mer, Y_n' , is given by the integral of the positive terms of equation 5..

$$\begin{aligned} Y_n' &= \frac{n}{N} \int_0^1 2(1-s)^{n-1} + 2s(N-n-1)(1-s)^{n-1} ds \\ &= \frac{2}{n+1} \end{aligned} \quad 6.$$

Thus the yield of n -mer is independent of the size or size-distribution of the original polymer.

Painter (1963) showed that, if all oligomers smaller than the n -mer are also resistant to attack, then the weight-fraction of $(n-1)$ -mer may be calculated from

$$Y'_{n-i} = \frac{2}{n-i+1} \prod_{j=0}^{i-1} \frac{n-j-1}{n-j+1}, \quad \begin{array}{l} n = 3, 4, 5 \dots \\ i = 1, 2, 3 \dots \end{array}$$

which simplifies to

$$Y'_{n-i} = \frac{2(n-i)}{n(n+1)} \quad \text{for } n > i. \quad 7.$$

Equation 7. gives theoretical yields of resistant oligomers after complete degradation of the polymer to oligomers of size n and smaller. If, however, only a fraction, x , of the polymer has been degraded this far, then the weight-fractions of the oligomers may be found from

$$Y'_{n-i} = \frac{2(n-i)}{n(n+1)} x. \quad 8.$$

Theoretical yields of maltodextrins produced by the random hydrolysis of amylose were calculated from equation 8., assuming a) that G_5 and $<G_5$ were not degraded further, i.e. $n = 5$, $i = 0-4$, or b) that G_6 and $<G_6$ were resistant to attack, i.e. $n = 6$, $i = 0-5$. The calculated yields are shown in Tables 5.16-5.18 and 5.20 [assumptions (11a) and (11b)].

For the pancreatic α -amylase, theoretical yields were calculated for a) G_3 and $<G_3$ resistant i.e. $n = 3$, $i = 0-2$ and b) G_4 and $<G_4$ stable i.e. $n = 4$, $i = 0-3$. These yields are shown in Table 5.19 [assumptions (11a) and (11b)].

A comparison of the experimental and theoretical values in Tables 5.16-5.20 indicates that the scheme of random degradation of amylose to give short resistant oligosaccharides does not adequately describe the action

of plant, bacterial or mammalian α -amylases. For the plant and bacterial enzymes, less G_4 and G_5 and more G_2 (plant enzymes) or G_3 (bacterial enzyme) and G_6 were found experimentally than predicted; for the mammalian amylase, there was (experimentally) an excess of G_2 and a deficiency of G_1 .

(iii) Theories making simple assumptions concerning the specificities of the α -amylases.

In view of the lack of success of the above theories, it was decided to attempt calculations making different simple assumptions regarding the specificities of the various types of α -amylases.

a) Bean, cereal and bacterial enzymes: The presence of a high yield of G_6 in the plant and bacterial enzyme digests may be explained by postulating that the enzymes cannot readily attack the 5 bonds nearest one end of a polysaccharide chain. By the theory of Bird and Hopkins (1954), these resistant linkages would be near the non-reducing chain-end. Then from the degradation of G_{12} , for example, the final yields of products would be $G_1 = 8.3\%$; $G_2 = 8.3\%$; $G_3 = 8.3\%$; $G_4 = 8.3\%$; $G_5 = 8.3\%$; $G_6 = 58.5\%$, i.e. there would be a high proportion of G_6 and equal amounts of G_1 - G_5 .

b) Cereal and bacterial enzymes: As little glucose was produced from amylose by the cereal and B. subtilis enzymes, an additional restriction may be made, that the bond at the

reducing chain-end is not readily hydrolysed. From G_{12} , the final proportions of oligosaccharides would be $G_1 = 0$; $G_2 = 10.5\%$; $G_3 = 9.2\%$; $G_4 = 8.8\%$; $G_5 = 8.3\%$; $G_6 = 41.6\%$; $G_7 = 21.6\%$. There would, in this case, be no G_1 and high yields of G_6 and G_7 .

Neither of these two schemes accounts adequately for the actions of the plant and bacterial α -amylases, but the second scheme is more satisfactory as it predicts the formation of rather more G_2 and G_3 than G_4 and G_5 .

c) Cereal enzymes: Bird and Hopkins (1954) suggested that malted barley α -amylase hydrolysed the second bond from a reducing chain-end more readily than the other linkages of amylose. If it is postulated, then, that (1) the first 5 bonds at the non-reducing end of a saccharide molecule are resistant to attack by cereal α -amylases, (2) the bond at the reducing chain-end is half as likely to be attacked as other linkages, and (3) the penultimate bond at the reducing end is twice as readily hydrolysed as all others, the final yields from G_{14} would be $G_1 = 5.9\%$; $G_2 = 13.7\%$; $G_3 = 6.7\%$; $G_4 = 6.7\%$; $G_5 = 6.8\%$; $G_6 = 60.2\%$. At a stage when the degradation is incomplete and the mixtures contain 27 or 20% of G_7 , the proportions of sugars would be as shown in Table 5.18 [assumption (iii)], if G_7 gives, on hydrolysis, 14.3% G_1 and 85.7% G_6 .

These figures agree fairly well with the experimental results for the malted wheat α -amylase.

d) Soya- and broad-bean enzymes: A similar scheme may be used for the bean enzymes, if the bond at the reducing end

of a molecule is not considered more resistant than other linkages, but the bond third from the reducing end is as easily attacked as the penultimate linkage. From G_{14} , the yields of saccharides would be $G_1 = 7.2\%$; $G_2 = 11.6\%$; $G_3 = 11.2\%$; $G_4 = 6.0\%$; $G_5 = 6.2\%$; $G_6 = 57.8\%$. At a stage of degradation when 25 or 29% G_7 is present, the composition of the mixtures would be as shown in Tables 5.16 and 5.17 [assumption (iii)].

The theoretical values compare favourably with those obtained experimentally for the soya-bean and broad-bean α -amylases.

In schemes c) and d) above, it was assumed that (1) G_6 and $\langle G_6$ are not degraded further by the plant amylases, and (2) G_7 gives only G_1 and G_6 . The experimental yields of G_6 would then ^{be} expected to be less than the predicted values, and this was found, in fact, to be the case for all the plant enzymes.

e) Pancreatic enzyme: Bird and Hopkins (1954) deduced that a mammalian α -amylase (from human saliva) did not readily attack the two bonds nearest the non-reducing end of a glucosidic chain or the bond at the reducing end. They also considered that the linkage second from the reducing end was susceptible to enzymic action. If it is assumed that this bond is three times more likely to be cut than others, then the products from G_{14} would be $G_1 = 0$; $G_2 = 31.3\%$; $G_3 = 37.5\%$ and $G_4 = 31.2\%$. If G_4 is slowly hydrolysed to G_2 , the yields of oligosaccharides would be as shown in Table 5.19 [assumption (iii)], at the stages when 21 and 10%

of G_4 are present.

The theoretical values are in good agreement with the results for porcine pancreatic α -amylase. As glucose was found in the digests, the bond at one chain-end cannot be completely resistant to amylolytic attack. This bond is probably situated at the reducing end of an oligosaccharide molecule.

f) Bacterial enzyme: For the α -amylase of B. subtilis, Bird and Hopkins (1954) postulated an action similar to that of the malted barley enzyme, with the third (instead of the second) bond from the reducing end of a glucosidic chain susceptible to attack. If this bond is four times more labile than other bonds, other restrictions being the same as for the cereal enzymes (scheme c, above), the following sugars would be obtained from G_{14} : $G_1 = 5.8\%$; $G_2 = 7.6\%$; $G_3 = 19.4\%$; $G_4 = 5.4\%$; $G_5 = 5.9\%$; $G_6 = 56.1\%$. Before complete degradation to G_6 and $<G_6$, the yields of sugars would be as shown in Table 5.20 [assumption (iii)], for 19 or 10% of G_7 present in the mixtures.

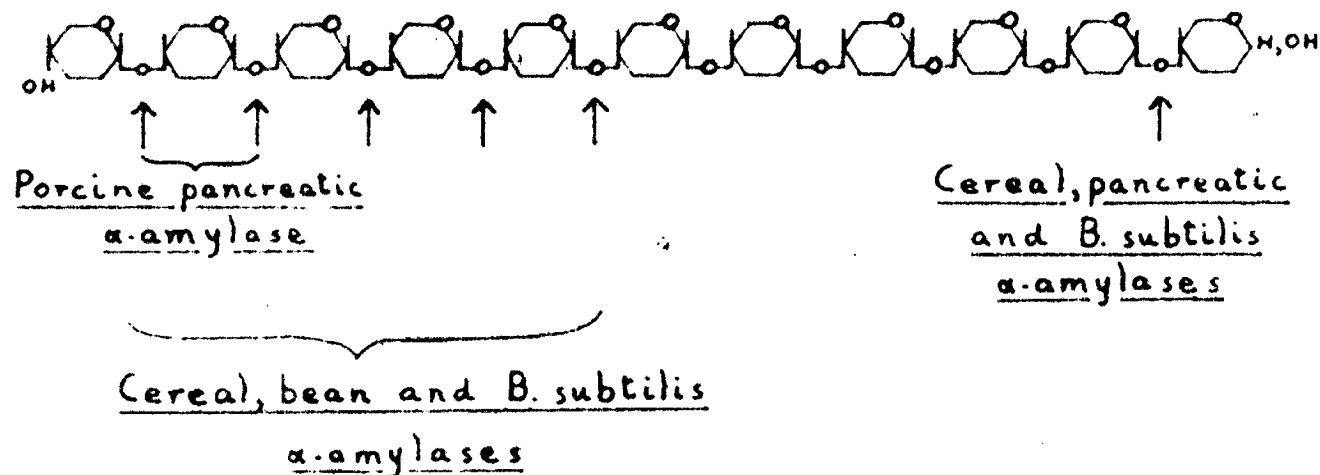
A comparison of these values with the experimental results show that more G_5 and less G_6 are formed than predicted. This would be expected, as no account was taken of the decomposition [shown by Robyt and French (1963)] of G_7 to give G_2 and G_5 , as well as G_1 and G_6 .

Robyt and French (1963) proposed that the α -amylase of B. subtilis forms G_6 preferentially from longer saccharides. Scheme d) shows, however, that a high yield of G_6 may be obtained if the sugar is considered to be the largest

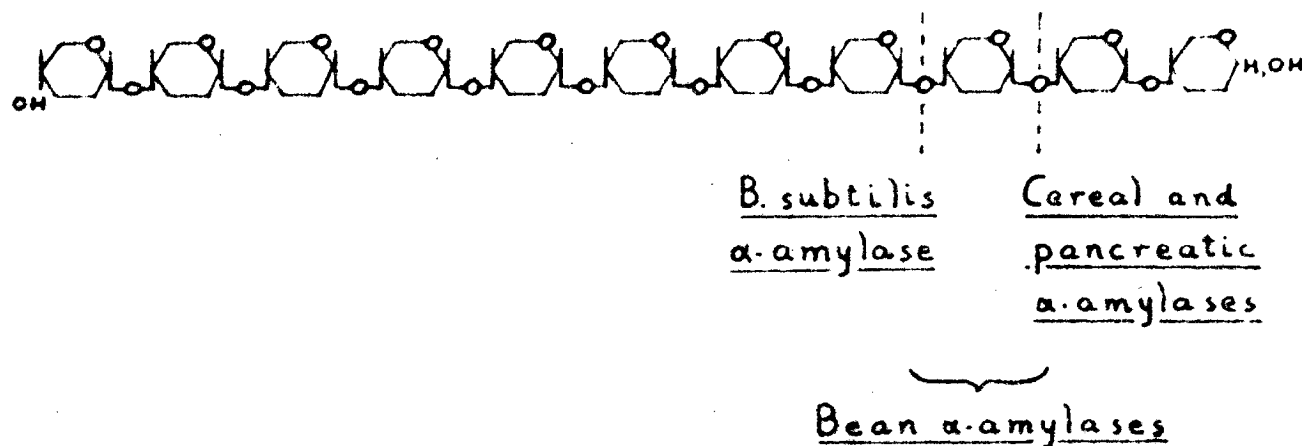
FIG. 5.5


Bonds in α -1:4 glucans attacked non-randomly by α -amylases

Resistant bonds — indicated by \uparrow



Labile bonds — indicated by \vdots



 = glucose ring

oligosaccharide resistant to α -amylolysis; thus it is not necessary to postulate a preferential formation to explain a high yield.

Concluding comments:

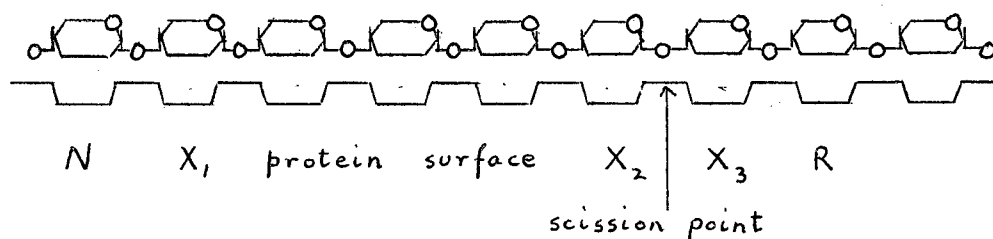
The products formed by the α -amylolysis of linear molecules may, therefore, be explained, if certain bonds near chain ends are considered more resistant or labile than others to enzymic attack. The resistant and labile bonds are shown in Fig. 5.5.

The apparent random nature of amylolysis in the initial stages is not inconsistent with the schemes above. In an amylose molecule of degree of polymerisation ca. 3,000, there are only 7 or 8 bonds which are resistant, or very susceptible, to attack by plant α -amylases, leaving at least 2,991 linkages which may be hydrolysed randomly. Initially, therefore, the degradation appears to be random, but as amylolysis proceeds, the proportion of chain-ends increases, and so the reaction rate slows down and non-random attack predominates.

Hypothetical scheme for α -amylase action

The variation in action pattern for different types of α -amylases makes it necessary to postulate a hypothetical scheme for each group of enzymes. A scheme for the cereal α -amylases is given below:

Sites 1. 2. 3. 4. 5. 6. 7. 8. 9.



X_1, X_2 = sites unfavourable for binding a non-reducing end-group.

X_3 = site unfavourable for binding a reducing end-group.

R = site favourable for binding any glucosidic residue of a chain, especially a reducing end-group.

N = site favourable for binding any glucosidic residue, especially a non-reducing end-group.

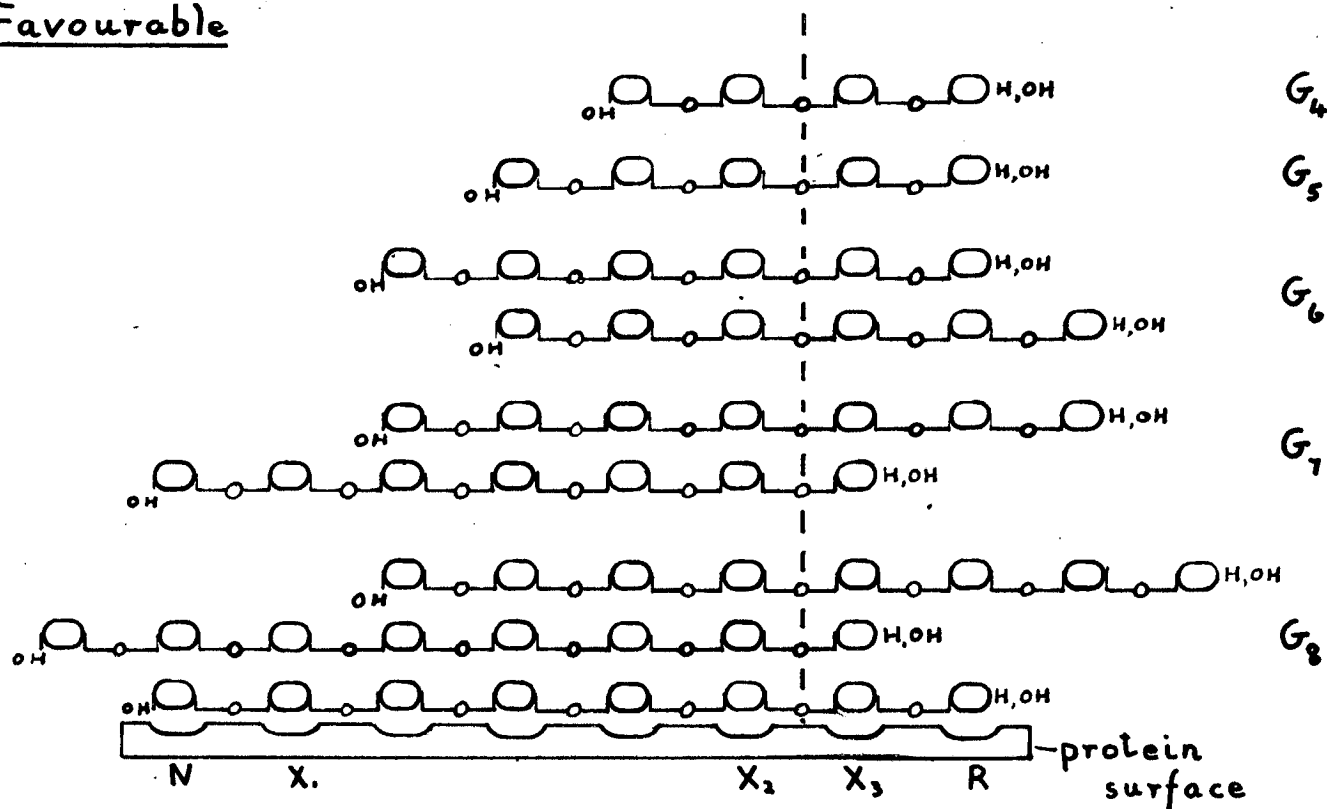
It is considered that the stereochemical arrangement of the enzyme active centre (containing substrate-binding groups, and the pH-dependent nucleophilic and electrophilic groups which catalyse the scission of the glucosidic bond) is such that a chain of at least 8 glucosidic units is required for easy binding; two of these units would be bound on one side of the scission point and six on the other.

The distribution of resistant and labile bonds shown in Fig. 5.5³ suggests that there may be preferential binding of reducing and non-reducing end-groups at sites R and N, respectively, and that binding of end-groups may be more difficult at sites X_1 - X_3 . These sites do not cause equal reductions of the ease of combination of enzyme with substrate; X_1 has a greater effect than X_3 . In the cases where the influence of a favourable site opposes that of an unfavourable site, the effect of X_1 overrides that of R, while the effect of N is capable of overriding that of X_3 . The difficulty of binding at sites X_1, X_2 or X_3 may be caused by steric factors involving the C1-OH or the C4-OH of the glucose units.

FIG. 5.6

Interactions of maltodextrins with hypothetical active site

Favourable



Unfavourable

○ = a glucose ring

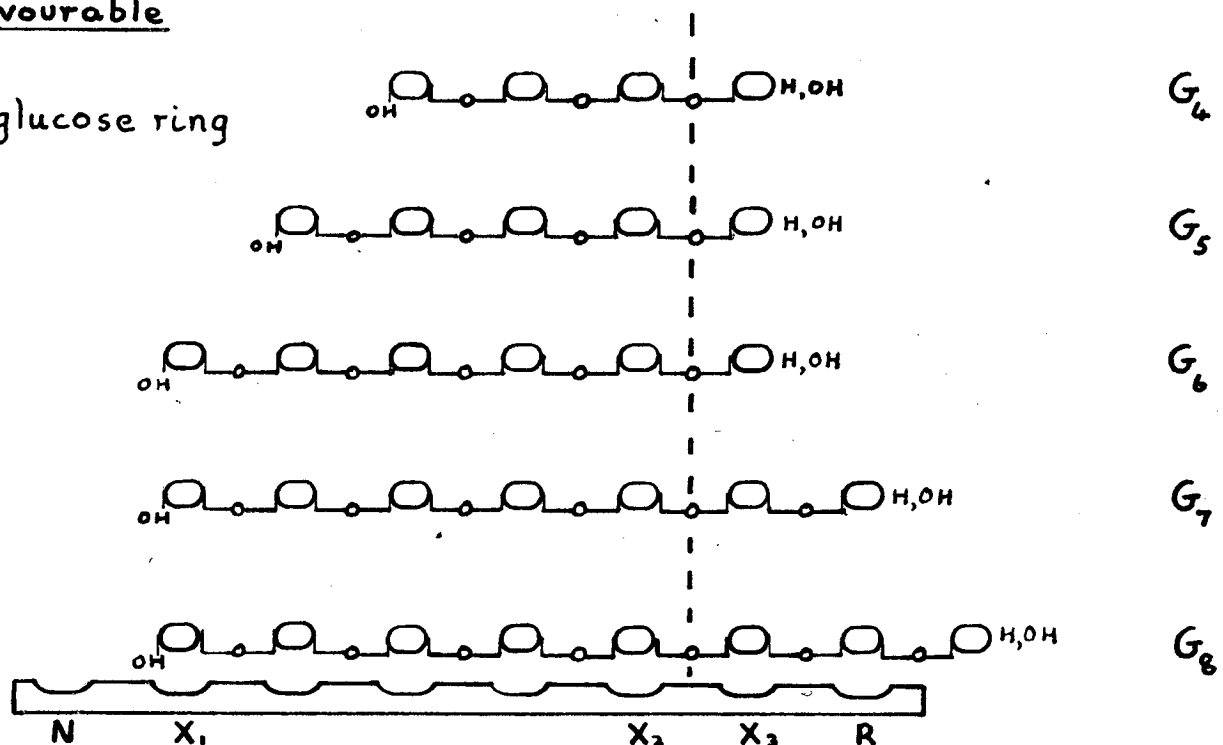


Fig. 5.6 shows the favourable and unfavourable degradation products which might be expected from various oligosaccharides. The scheme accounts satisfactorily for the experimental results.

Substrate molecules are considered to be bound most easily if they are long enough to combine with the protein at sites N and R simultaneously. Shorter molecules, i.e. G_7 and smaller oligosaccharides, would be bound to the enzyme with greater difficulty, and so hydrolysis of these sugars would be expected to be very slow. The ease of binding is thought to be proportional to the length of the maltodextrin chain. Thus G_7 would be attacked faster than G_6 , which, in turn, would react more readily than G_5 or G_4 .

Similar types of schemes may be postulated for other α -amylases, with different distributions of the favourable and unfavourable binding sites.

For the bean enzymes, sites N, X_1 , X_2 and R may be situated as above, while site 7 (X_3 for the cereal enzymes) may not be specifically unfavourable for binding a reducing end-group. There may, however, be additional favourable binding at site 9.

The active centre of B. subtilis α -amylase may be represented by a scheme in which sites N, X_2 and X_3 are situated as above, but sites 2 and 8 (X_1 and R for the cereal enzymes) are not specifically unfavourable or favourable for binding. Site 9 may be favourable for

binding a reducing end-group. In this case, the substrate chain must consist of at least 9 glucosidic units for easy combination with the enzyme.

The arrangement of binding groups at the catalytically active centre of the porcine pancreatic enzyme may be rather different to those of the plant and bacterial α -amylases, as the mammalian enzyme appears to attack bonds fairly near a non-reducing chain-end with little difficulty. Sites X_2 , X_3 and R may be in positions similar to those shown for the cereal enzymes. Sites 1 and 2 (N and X_1 for the cereal amylases) may not be specifically favourable or unfavourable for binding, although there may be a favourable binding site at position 4. A chain-length of at least 5 glucosidic units would be required for easy enzyme-substrate combination.

As plant and bacterial enzymes attack long substrate molecules very much more quickly than short ones [^{81, 86}Schwimmer (1950), Robyt and French (1963)], it is possible that there are other binding sites in the enzymes, situated further away from the "scission point" than those discussed above.

These hypothetical schemes are based on the results of studies of the α -amylolysis of amylose and oligosaccharides. It is realised that, as more information becomes available concerning the nature of the substrate-enzyme interactions, and the arrangement of amino acids in the enzyme molecules, some modification of the schemes may be necessary.

It would be of interest to obtain comparable results for the α -amylolysis of amylopectin, but more information regarding the detailed structure of the polysaccharide is required before this could be carried out effectively.

Summary

A brief account is given of the actions of the more important starch-degrading enzymes. The general experimental techniques used in this study are outlined, and a method for measuring α -amylolytic activity in the presence of β -amylase is described.

α -Amylases have been isolated from soya- and broad-beans, oats, rye, wheat, germinated barley and germinated wheat. These amylases have been purified free from other starch-metabolising enzymes by a procedure involving acetone fractionation and heat-treatment in the presence of an excess of calcium ions. The method used to prepare the bean and germinated cereal α -amylases includes the formation of an insoluble glycogen-enzyme complex.

The effect of various salts and of temperature on the activity of the α -amylases from oats, rye and wheat has been examined. Viscometric techniques have been used to study the activity of these cereal enzymes at pH 3.6 and in the presence of ethylene diamine tetraacetate and trypsin. Variation of amylolytic activity with pH and with modification of the enzymes has enabled the nature of the amino acids at the catalytically active centres of the cereal and bean amylases to be investigated. These amino acids are thought to contain free carboxyl, imidazolium and amino groups, but not sulphydryl groupings.

The molecular sizes of the α -amylases of germinated and dormant wheat and germinated barley have been compared by gel-filtration techniques. The results indicate that the three enzymes are of similar molecular size, i.e. no large change in molecular weight of a cereal α -amylase takes place on germination of the cereal.

The action-patterns of several α -amylases on linear substrates have been investigated. The kinetics of the initial stage of the α -amylolysis of amylose by the enzymes from oats, rye and wheat have been studied viscometrically. The results indicate that the process is random. However, paper-chromatographic investigations of the production of maltodextrins from amylose and the hydrolysis of small oligosaccharides have shown that, at later stages, non-random attack predominates. This has been confirmed by determining the yields of maltodextrins produced from amylose after the achroic limit. Plant, bacterial and mammalian α -amylases have been compared, and four types of action-pattern have been found, corresponding to the enzymes from beans, cereals, B. subtilis and porcine pancreas. Little change appears to take place in the action of a cereal α -amylase on germination of the cereal.

Theories for the action-patterns of the different types of α -amylases have been proposed to account for the experimental results. The enzymes are thought to act in a non-random manner on α -1:4 bonds near the chain-ends of substrate molecules, but hydrolyse randomly all other

α -1:4 bonds. Hypothetical schemes are presented for the active centres of bean, cereal, bacterial and mammalian α -amylases.

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STUDIES ON STARCH-DEGRADING ENZYMES

PART II* THE Z-ENZYME FROM SOYA BEANS; PURIFICATION AND PROPERTIES

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INTRODUCTION

Z-Enzyme was first found to be associated with soya-bean β -amylase when amylose, which was incompletely hydrolysed by crystalline sweet-potato β -amylase, was *completely* degraded into maltose by the "purified" soya-bean enzyme^{1,2}. Most samples of amylose are now known to contain some type of barrier to the action of pure β -amylase³⁻⁵. Peat *et al.*^{2,6} initially suggested that Z-enzyme specifically removed this structural feature. However, by preferentially inhibiting the β -amylase, we established that Z-enzyme was α -amylolytic in character, and consequently non-specific with regard to its action on the barrier in amylose⁷. This general conclusion has been confirmed by later workers^{8,9}.

In this paper, we extend our earlier observations on Z-enzyme, and report a convenient method for isolating and extensively purifying this enzyme from soya beans. This has enabled us to make the first detailed studies of the properties of the purified enzyme.

EXPERIMENTAL

General analytical methods

Routine determinations of protein concentrations were made from absorption measurements at 280 m μ , the method being calibrated by micro-Kjeldahl estimations. The concentration of polysaccharide solutions was estimated by hydrolysis, and titration of the liberated glucose with alkaline ferricyanide³. Concentrations of reducing sugar in enzymic digests were estimated by the same reagent. β -Amylolysis limits, $[\beta]$, were carried out as described earlier³, except that crystalline sweet-potato β -amylase (Worthington Biochemical Corporation, New Jersey, U.S.A.) was used. (This enzyme was shown to be free from Z-enzyme activity.) The technique for measuring the limiting-viscosity number, $[\eta]$, has been detailed elsewhere¹⁰; 0.2 M potassium hydroxide was used as solvent, and measurements were made at 25°.

* For Part I, see ref 31.

Substrates

Soluble starch ('Analar', B.D.H.) was used for estimation of β -amylase activity. Amylose ($[\eta] = 500$, $[\beta] = 80$) and amylopectin ($[\eta] = 180$, $[\beta] = 56$) were prepared³ from a dispersion of potato starch (var. Redskin). Linear amylose ($[\eta] = 260$, $[\beta] = 100$) was obtained by aqueous leaching of potato-starch granules³. Glycogen ($[\beta] = 45$) was extracted from rabbit livers with cold trichloroacetic acid¹¹. β -Limit dextrins from amylopectin and glycogen were obtained by dialysis and freeze-drying of the appropriate digests.

Digest conditions

Unless otherwise stated, digests were carried out at 35°, and the pH was controlled by acetate buffer (0.2 M, pH 5.5).

Measurement of enzymic activity

β -Amylase. Measurement was made of the amount of maltose produced in a 1 ml portion of a digest containing starch solution (0.6%, 25 ml), buffer (4 ml, pH 3.6), and enzyme (1 ml), after incubation for 30 min. Activities were then expressed as mg of maltose produced per mg of protein.

Z-Enzyme. A modification of the procedure devised by Briggs¹² was employed. The β -limit dextrin from amylopectin was used as substrate, and the "time reference point¹²" was chosen as the time when the corrected absorption value (A.V.) had fallen to 2.00 colorimeter units.

Digests were prepared from buffer (1 ml, pH 5.5), dextrin (amylopectin β -limit) solution (2 ml, 0.6%), enzyme solution, and water to give a total volume of 8 ml. Aliquot portions (2 ml) were removed at intervals and treated with iodine (1 ml, 0.2% in 2% potassium iodide) and hydrochloric acid (0.2 ml, 5 M), in a total volume of 50 ml. The A.V. was then measured at 540 m μ in an EEL-colorimeter (filter No. 625). Activities were expressed¹² as iodine-dextrin-colour units/mg of protein/ml of digest.

Preparation of Z-enzyme

(a) *Initial extraction and fractionation.* Dry, defatted, finely-ground, soya-bean flour (300 g) was shaken with calcium chloride solution (0.2%, 1500 ml) for 4 h at 18°. After centrifugation at 900 g, the resultant supernatant liquor (ca. 40 mg of protein/ml) was cooled to 0°. Acetone at -5° was added to a concentration of 10% (v/v). The temperature of the mixture was then lowered to -5° and cold acetone was added slowly, with continuous stirring. Protein fractions obtained by centrifugation (at -5°, 1100 g) were air-dried to remove excess of acetone, and suspended in water at 2°; any insoluble residue was removed by centrifugation. The fractions were characterized, and a typical result is shown below:

Acetone concentration (v/v)	0	20	30	35	40	45	50	55	60
Fraction	0	1	2	3	4	5	6	7	8
Specific activity of Z-enzyme	6.4	5.0	5.3	7.8	15.4	20.0	35.0	36.0	4.6
Specific activity of β -amylase	7.6	6.7	7.0	8.5	13.0	12.0	12.0	9.0	5.0

(b) *Removal of β -amylase; preliminary experiments.* Portions of fraction 5 were heated on a water-bath at 70° (i) in the presence of added calcium acetate (2 mg/ml), and (ii) without additional calcium ions. The protein content, β -amylase, and Z-enzyme activities of aliquot portions of the cooled, centrifuged (1500 g) digests were then measured at appropriate intervals. The results in Fig. 1 indicate

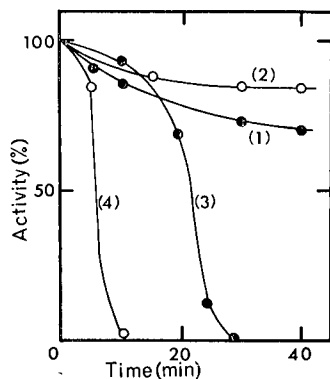


Fig. 1. Effect of heat treatment at 70° and pH 5.5 on the activity of Z-enzyme and β -amylase in soya-bean extract: (1) Z-enzyme in the absence of Ca^{2+} ; (2) Z-enzyme in the presence of Ca^{2+} (10^{-2} M); (3) β -amylase in the absence of Ca^{2+} ; (4) β -amylase in the presence of Ca^{2+} (10^{-2} M).

that, although the activity of the crude Z-enzyme preparation was remarkably stable at pH 5.5 and 70° (curve 1), the stability was further increased by the addition of calcium ions (curve 2). In the presence of this metal ion, the β -amylase was almost completely deactivated in 10 min under these conditions (curve 4).

Removal of β -amylase; final procedure. Uniform heat-treatment on the water-bath was ensured by heating standard portions (10–15 ml) of the enzyme fractions. To such portions of fraction 7 were added calcium acetate (20 mg), and acetate buffer to bring the pH to 5.5. The mixture was then maintained at 70° for 20 min, cooled, and centrifuged at 1500 g. Resultant supernatant liquors were combined. Typical specific activities were as below:

	β -Amylase activity	Z-Enzyme activity
Initially	9	36
After heat treatment	0	51

This heat treatment was shown to irreversibly deactivate the β -amylase.

(c) *Fractionation with acetone.* The heat-treated enzyme solution was sub-fractionated with acetone at -5° . Typical results for the protein fractions obtained were as below:

Acetone concentration (v/v)	0	35	42	47	52	57
Fraction	7	7.1	7.2	7.3	7.4	7.5
Specific activity of Z-enzyme	51	6.8	33	90	450	92

(d) *Glycogen-complex formation.* The acetone-precipitated fractions were finally purified as the glycogen complex, by the method of Loyter and Schramm¹³.

Ethanol to 40% v/v was slowly added with stirring to the enzyme solution at 2°. After 15 min, any precipitated protein was removed at 1100 g. Phosphate buffer (0.5 ml/10 ml of enzyme solution, pH 8.0, 0.2 M) and glycogen (2%, 0.2 ml/10 ml of enzyme) were then added to the enzyme in 40% v/v ethanol. The suspension was stirred for 10 min, and then centrifuged at 1100 g to yield a precipitate which was suspended in phosphate buffer (pH 6.7, 0.02 M). The suspension was maintained at 35° for 6 h to aid digestion of contaminating glycogen, and centrifuged at 1500 g. The resultant solution was cooled to 2° and treated with excess of acetone, and the precipitated material redissolved in water at 2°. Enzyme solutions were found to retain their specific activity (*ca.* 800) at this temperature for several weeks.

Effect of temperature and pH

In experiments where the effect of temperature on activity was studied, digests at pH 5.5 were pre-incubated to temperature equilibrium before enzyme was added. In stability experiments at pH 5.5, the enzyme solution and buffer were maintained at the appropriate temperature for 1 h, and then cooled to 35° before addition of the dextrin. Resultant activities were compared with those obtained at 35°, without prior incubation. The pH dependence of activity at 35° was obtained using McIlvaine's standard buffer solutions. The effect of pH on enzyme stability was determined by maintaining the enzyme and buffer at 20° for 75 min. Digests were then brought to pH 5.5 and incubated with dextrin at 35°, and the activities determined.

Activity at pH 3.6. Digests were prepared as follows:

- (a) Buffer (2 ml, pH 5.5) + enzyme (0.2 ml) + amylose (20 ml, 3 mg/ml)
- (b) Buffer (2 ml, pH 5.5) + amylose (20 ml, 3 mg/ml)
- (c) Buffer (2 ml, pH 3.6) + enzyme (0.2 ml) + amylose (20 ml, 3 mg/ml)
- (d) Buffer (2 ml, pH 3.6) + enzyme (0.2 ml); incubated for 2 h at 20° before amylose (20 ml, 3 mg/ml) was added.

After incubation at 35° for 24 h, the digests were heated on a boiling water-bath for 5 min, cooled, and filtered, and the amylose product precipitated with excess of butan-1-ol. No butan-1-ol complex was obtained in digest (a). For the products from digests (b)–(d), $[\eta]$ was measured.

Effect on amylose-viscosity of Z-enzyme pretreated with mercuric and calcium chlorides

Digests of enzyme and buffer (pH 5.5) were prepared containing (e) 10^{-3} M mercuric chloride, (f) 10^{-4} M mercuric chloride, (g) 10^{-3} M calcium chloride, and (h) no additional salts. After incubation at 20° for 2 h, the solutions were added to equal volumes of the same amylose solution (3 mg/ml) and incubated at 35°.

Butan-1-ol was added to digests (g) and (h) after 1 h, and to digests (e) and (f) after 24 h; $[\eta]$ of the amylose products was then determined.

Effect of ethylenediaminetetra-acetate (EDTA) and trypsin

Digests (i)–(n) were prepared by adding to 10 ml portions of digests at pH 6.6 (McIlvaine's buffer); for (i) and (j), EDTA (0.1 ml, 10^{-1} M) and trypsin (0.1 ml, 0.4 mg); for (k) and (l), EDTA (0.1 ml, 10^{-1} M); for (m) and (n), trypsin (0.1 ml, 0.4 mg). A control digest (o) was also prepared. After incubation at 20° for 12 h, calcium chloride (1 ml, 1 M) was added to digests (i), (k), and (m), and coagulated protein removed by centrifugation. Equal volumes of the same amylose solution (3 mg/ml) were added to all of the digests, which were then incubated at 35° for 24 h. Excess of butan-1-ol was then added; no amylose was precipitated from digests (m) and (o). For the amylose products from the other digests, $[\eta]$ was determined.

Modification of Z-enzyme

*Coupling with p-diazobenzenesulphonic acid*¹⁴. Phosphate buffer (1 ml, pH 8.2) and p-diazobenzenesulphonic acid (0.1 ml, 0.6%) were added to Z-enzyme (2 ml, activity = 2 units/ml). A control digest was set up containing sulphanilic acid (0.1 ml, 0.6%). The mixtures were left at 18° for 1 h, dialysed at 2° against calcium acetate (0.2%, 3×200 ml), and centrifuged. Activities were then determined in the usual way.

*Acetylation*¹⁴. Sodium acetate (250 mg) was added to Z-enzyme (1 ml, activity = 2 units/ml) at 0°, and then acetic anhydride (0.03 ml) was added. After 1 h, the mixture was dialysed as above. A control was prepared without the anhydride, and the activities of both digests were determined.

RESULTS AND DISCUSSION

Isolation and purification of Z-enzyme

Z-Enzyme was first characterized^{1,2} as the enzyme which — associated with, and acting in conjunction with, soya-bean β -amylase — would completely degrade any amylose at pH 4.6, but which was itself inhibited completely at pH 3.6. This latter behaviour, in conjunction with the fact that classical reducing-power tests were negative, led to the suggestion that Z-enzyme was not an α -amylase. The more sensitive, physical techniques of viscosity and light-scattering were necessary to establish the α -amylolytic character of the enzyme⁷. In this work, therefore, we have used procedures applicable to α -amylases to isolate and purify the Z-enzyme in soya beans.

Initially, a method for measuring Z-enzyme activity in the presence of the contaminating β -amylase had to be developed. Methods involving estimation of reducing power¹⁵, fall in viscosity¹⁶, or decrease in starch-iodine stain¹⁷ are all influenced by concurrent β -activity. However, a modification of Briggs' method¹²,

in which we used β -limit dextrin from amylopectin as the substrate, was satisfactory. Any fall in iodine-staining power of this substrate must be due to α -amylolysis. (Although in the presence of excess of β -amylase, α -amylolytic activity will be followed by β -amylolysis, the effect of this on the iodine-staining ability of the dextrin was shown to be small). This method, which is based on the assay of Sandstedt *et al.*¹⁸, measures activity as the reciprocal of the time taken to decrease the dextrin-iodine stain by a standard amount. Here, the standard graph, from which subsequent Z-enzyme activities were calculated, was constructed by measuring the effect of salivary α -amylase on the limit dextrin. (It was later found that hydrolyses of the dextrin by salivary α -amylase and the purified Z-enzyme were very similar in the initial stages).

Preliminary experiments showed that fractionation of the soya-bean protein by ammonium sulphate and alcohol was not successful, little separation of β -amylase and Z-enzyme activities being achieved. (All β -amylase activities were determined at pH 3.6 to inhibit the Z-enzyme.) A successful separation was made, however, by the use of acetone at low temperatures. Heat-treatment was attempted to remove the contaminating β -amylase, as it has been shown^{19,20} that β -amylase activity can be preferentially removed from malted-barley α -amylase preparations by heating the enzyme mixture at 70°, in the presence of calcium ions. Experiments showed (Fig. 1) that a comparable heat-treatment at 70° and pH 5.5 preferentially, and irreversibly, deactivates the β -amylase in the Z-enzyme/ β -amylase fractions. After this removal of the β -amylase, further inert protein was removed by a second fractionation with acetone. Finally, the specific activity of the Z-enzyme was nearly doubled by the formation of a glycogen complex. The latter procedure has been suggested^{13,21} as a general method for preparing α -amylases of very high activity.

The purification procedure increased the overall specific activity of Z-enzyme by a factor of *ca.* 150. Maltase, laminarase, and cellobiase were absent, as shown by digestion with the appropriate substrate, followed by paper chromatographic analysis. Similarly, incubation of the enzyme preparation with maltotriose for 72 h showed the presence of trace amounts of glucose and maltose, probably arising from the slow action of Z-enzyme itself; D-enzyme was absent.

Effect of temperature on activity and stability

The temperature of maximal activity of Z-enzyme is *ca.* 55° (see Fig. 2*a*). The purified enzyme lost only *ca.* 10% of its original activity after 1 h at 50°, but there was then a very rapid decrease between 55 and 60°. In the heat-treatment stage of the preparation procedure, the Z-enzyme appears to be stabilized by contaminating protein. An Arrhenius plot of the temperature dependence of the activity is shown in Fig. 2*b*; the apparent heat of activation varies from 14 kcal at 9°, to 6 kcal at 25°, and is zero at 55°.

Effect of pH on activity and stability

The effect of pH on the enzymic activity is shown in Fig. 3*b*, where the ratio

(activity at a given pH, V_{H^+})/(maximum activity at the optimum pH, V_{max}) is plotted against the pH. A variation in substrate concentration did not affect the shape and position of the curve. The results have been analysed, using the scheme shown in

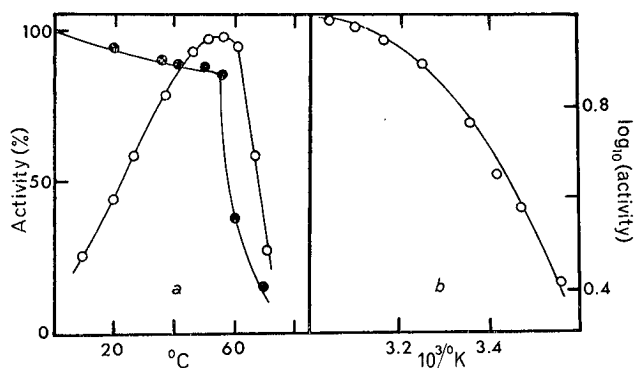


Fig. 2. (a) Effect of temperature on the activity (-○-) and stability (-●-) of Z-enzyme; (b) Arrhenius plot of temperature dependence of activity.

Fig. 4, for an enzyme with two ionizable groups²²⁻²⁵. If (i) the form EHS is assumed to be the only one of the three enzyme-substrate complexes capable of reacting to give the products, and (ii) the ionization of the two groups concerned is considered

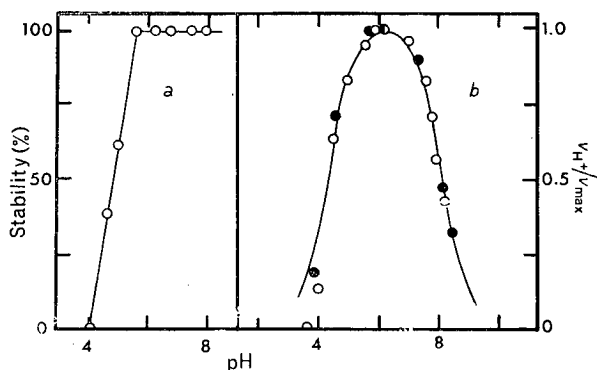


Fig. 3. Effect of pH on the stability (a) and activity (b) of Z-enzyme: (-○-) substrate concentration = 0.7 mg/ml; (-●-) substrate concentration = 2.8 mg/ml. The solid line represents the theoretical curve discussed in the text.

to be unaffected by substrate binding, i.e. $K'_a = K_a$ and $K'_b = K_b$, it can be readily shown²⁵ that

$$\frac{V_{H^+}}{V_{max}} = \frac{1 + 2\sqrt{K_a/K_b}}{1 + K_a/[H^+] + [H^+]/K_b}$$

i.e. V_{H^+}/V_{max} is a function which is independent of the substrate concentration. As our experimental data showed such independence, the function and experimental

points in Fig. 3*b* were used to calculate K_a and K_b . Values of $pK_a = 8.15$, and $pK_b = 4.3$, were found. The solid line in Fig. 3*b* represents the function V_H/V_{\max} calculated from these values. Essentially, the experimental points lie on this theoretical curve, except at pH 4.0 and below, where irreversible denaturation of the enzyme may be occurring.

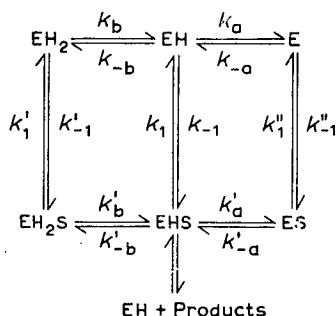


Fig. 4. Hypothetical scheme for the ionization and reaction of the enzyme; E = enzyme, H = proton S = substrate. Velocity constants (k) are as indicated. Ionization constants of the enzyme (K_a , K_b) and the enzyme/substrate complex (K'_a , K'_b) are defined as:

$$K_a = k_a/k_{-a}, \quad K_b = k_b/k_{-b} \\ K'_a = k'_a/k'_{-a}, \quad K'_b = k'_b/k'_{-b}.$$

The stability of Z-enzyme at various pH values is shown in Fig. 3*a*. Although these results suggest that all experimental values obtained below pH 5.5 in Fig. 3*b* should lie beneath the theoretical curve, the two sets of data are not directly comparable; in the stability experiments, the enzyme was incubated in the absence of substrate before the activity was determined, and hence the stabilizing effect of an enzyme-substrate complex was not present, as it was for the results in Fig. 3*b*.

Nature of the active centres in Z-enzyme

The nature of the ionizing groups under consideration may be inferred from the pK-values; the group with pK 4.3 is probably a carboxylic acid²⁶, whilst that with pK 8.15 is likely to be an ammonium group²⁶. However, there is the possibility that interaction with an anion may have displaced the pH-activity curve²⁷, in which case the ionizing group might be an imidazolium group (as in histidine)^{25,26}. As the behaviour of the enzyme can be explained in terms of the fact that the ionization is unaffected by binding with substrate, these groups may be involved in the breakdown of the enzyme-substrate complex to give the reaction products, rather than in the formation of a complex.

In order to investigate the nature of the group with pK = 8.15, digests were prepared incorporating (i) iodine (3×10^{-6} M), and (ii) sodium *p*-chloromercuribenzoate (3×10^{-5} M). In digest (i), inhibition was complete, whilst in (ii) there was no change in enzyme activity. Since iodine reacts preferentially with histidine, tyrosine, and sulphydryl groups, whilst the sodium salt only reacts with sulphydryl

groups, the results suggest that histidine and/or tyrosine are necessary for activity of Z-enzyme. This was confirmed when coupling of the enzyme with *p*-diazobenzene-sulphonic acid — a reagent which combines primarily with tyrosine and histidine residues in proteins — diminished the activity by 95%. Another reagent, which is fairly specific for free amino groups in proteins, although it also reacts with free sulphhydryl groups and, in some cases, free phenolic groups, is acetic anhydride. Acetylation reduced the activity of the Z-enzyme by 75%, suggesting that free amino groups are necessary. However, insufficient enzyme was available for an estimation of the degree of acetylation, or of the types of group acetylated. Thus diminished activity of the enzyme may have been caused by partial acylation of tyrosine residues.

TABLE I

ACTIVITY OF Z-ENZYME AT pH 3.6

Digest	(a)	(b)	(c)	(d)
Conditions ^a [η] of amylose, after incubation	pH 5.5 10 ^b	Control 510	pH 3.6; directly 400	pH 3.6; pre-incubation 500

^aSee Experimental section (p.232).^b[η] too small to be measured accurately.

TABLE II

EFFECT OF VARIOUS REAGENTS ON ACTIVITY OF Z-ENZYME

% inhibition from iodine-staining measurements is quoted

Molarity	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶
Mercuric chloride	100	100	42	15
Potassium cyanide	0	0	0	0
Ammonium molybdate	20	0	0	0
Tryptophan	0	0	0	0
Ascorbic acid	100	90	70	42

Activity of Z-enzyme at pH 3.6

As the behaviour of Z-enzyme at pH 3.6 is of critical consequence, we made a careful study of the effect of this pH on the enzyme's activity by following changes in [η] of an amylose. This technique yields an extremely sensitive measure of any hydrolytic action. The results of these experiments are shown in Table I, where the negligible [η] of digest (a) shows the high rate of amylolytic degradation at pH 5.5. The difference in [η] between samples (b) and (d) is within experimental error, and shows that pre-incubation at pH 3.6 for 2 h completely destroys the Z-enzyme activity. There was, however, a significant decrease in the viscosity of sample (c), showing that the substrate had been hydrolysed before complete inhibition of the

enzyme had been achieved. Pre-incubation for at least 2 h at pH 3.6 is essential, therefore, before β -amylolysis limits can be obtained using β -amylase preparations which contain Z-enzyme.

Effect of various reagents on activity

The effect of various reagents on the activity of Z-enzyme is shown in Table II. Potassium cyanide and tryptophane do not affect the activity, whilst the negligible effect of ammonium molybdate shows that the activities of Z- and R-enzyme are distinct²⁸. Ascorbic acid (10^{-3} M) and mercuric chloride (10^{-4} M) are very efficient inhibitors of Z-enzyme activity. Table II also shows that quite extensive inhibition of Z-enzyme must have occurred at the concentration of mercuric chloride (1.5×10^{-6} M) used in our original work⁷. Complete inhibition by mercuric chloride is shown by the $[\eta]$ -values for digests (e) and (f) in Table III. This Table also shows, from a comparison of the $[\eta]$ -values for digests (g) and (h), that calcium chloride (10^{-3} M) did not activate the enzyme.

TABLE III

EFFECT ON AMYLOSE-VISCOSITY OF Z-ENZYME PRETREATED WITH MERCURIC AND CALCIUM CHLORIDES

Digest ^a	(e)	(f)	(g)	(h)
Pretreatment conditions at pH 5.5 ^a	Hg ²⁺ = 10^{-3} M	Hg ²⁺ = 10^{-4} M	Ca ²⁺ = 10^{-3} M	—
$[\eta]$ of amylose, after incubation ^b	500	500	80	75

^aSee Experimental section (p. 232).

^b $[\eta]$ of amylose = 510.

The α -amylolytic nature of Z-enzyme suggested that its action might be very dependent on the presence of calcium ions. The importance of this ion to α -amylase activity has been extensively studied by Fischer and his collaborators²⁹. Calcium ions may be effectively removed from aqueous solution by the chelating action of ethylenediaminetetra-acetate (EDTA). In our experiments, the effect of the presence of EDTA on Z-enzyme activity was followed by measuring the amylolysis of amylose by changes in $[\eta]$, as this method again provides the most sensitive measure of assay. The fall in viscosity [45%; Table IV, digest (l)] shows that although the enzyme is inhibited to some extent [compare, digest (o)], hydrolysis has taken place in the EDTA/Z-enzyme/amylose digest. This inhibition is largely reversible, because on addition of excess of calcium ions, the extent of hydrolysis is greatly increased [digest (k)]. The protease, trypsin, has little effect on the enzyme activity as shown by the large decrease in $[\eta]$ for the amylose in digest (n), although this fall is even larger in the presence of calcium ions [digest (m)]. However, under the combined action of EDTA and trypsin there was only a 10% fall in $[\eta]$ for the amylose sample. Thus a mixture of EDTA and trypsin is a more efficient inhibitor than EDTA by itself. This inhibition is not completely reversible as, on the addition of excess of calcium ions, the amylose in digest (i) was not degraded to the same

extent as that in (k). This effect may be due to protease attack on calcium-deficient protein molecules.

TABLE IV

EFFECT OF EDTA AND TRYPSIN ON Z-ENZYME ACTIVITY

Digest ^a	(i)	(j)	(k)	(l)	(m)	(n)	(o)
Initial conditions ^a	EDTA/trypsin	EDTA/trypsin	EDTA	EDTA	trypsin	trypsin	control
Final conditions ^a	+Ca ²⁺	—	+Ca ²⁺	—	+Ca ²⁺	—	—
[η] of amylose ^b	75	455	33	280	10 ^c	40	10 ^c

^aSee Experimental section (p. 233).

^b[η] of amylose = 510.

^c[η] too small to be measured accurately.

Action of Z-enzyme on various substrates

In our earlier work⁷, the hydrolysis of linear amylose by Z-enzyme was indicated by viscosity results, whilst attack on amylopectin and amylopectin limit-dextrin was shown by light-scattering measurements. The preparation of purified enzyme has now enabled the action on these substrates to be studied by classical iodine-staining and reducing-power measurements. Fig. 5 shows that all three substrates can be hydrolysed to the "achroic limit". In particular, it should be noted that the action of Z-enzyme on the β -limit dextrin from amylopectin is comparable to that of salivary α -amylase. This justifies the method of estimation of activity, whilst showing the similar nature of the two enzymes.

It is apparent that there are two distinct stages in the hydrolysis of linear amylose by Z-enzyme. There is first a rapid decrease in size of the amylose molecules, as shown by the fall in colour of the iodine stain. This is accompanied by an increase in reducing power of the solution. The second part of the reaction begins at the achroic limit of the amylose solution, and is characterized by a slow increase in reducing power of the solution. The discontinuity occurs at *ca.* 30% apparent conversion into maltose, and the disappearance of the iodine stain indicates that there are only small maltodextrins present at this stage in the reaction. However, it has to be stressed that the achroic point during α -amylolysis of amylose is not invariant, but depends entirely on the amylose-enzyme ratio³⁰.

The effect of Z-enzyme on the β -limit dextrin from glycogen is not yet known with certainty. Our earlier light-scattering measurements indicated that there is no attack on this substrate, whilst Cunningham *et al.*⁹ obtained an increase in reducing power of the digest, using very large quantities of enzyme and prolonged incubation. If limited α -amylolytic attack is occurring, a more sensitive measure of this can be obtained from the concurrent action of β -amylase and the purified Z-enzyme. Under our normal digest conditions, with the addition of β -amylase, we found a

2% apparent conversion into maltose after 24 h. This result indicates that purified Z-enzyme does cause degradation of glycogen limit-dextrin.

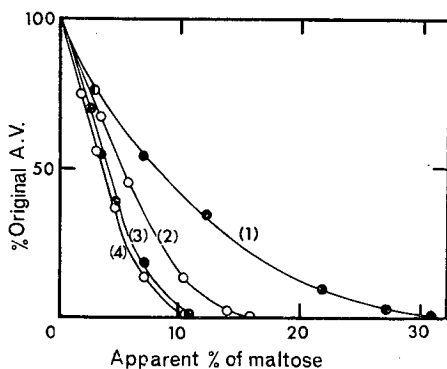


Fig. 5. Graph of % of original A.V. *versus* apparent conversion into maltose. (1) amylose/Z-enzyme; (2) amylopectin/Z-enzyme; (3) amylopectin β -limit dextrin/salivary α -amylase; (4) amylopectin β -limit dextrin/Z-enzyme.

The character of Z-enzyme

The above experiments have extended our original observations regarding the α -amylolytic character of Z-enzyme. The variation of activity and stability with temperature and pH are comparable to those of other α -amylases^{25,29}. The enzyme is irreversibly deactivated by pre-incubation at pH 3.6, but we have found that this behaviour is a characteristic of several other plant α -amylases³¹. Inhibition occurs in the presence of mercuric chloride, whilst calcium ions are essential for activity. As with other α -amylases²⁸, hydrolytic degradation of amylose, amylopectin, and glycogen occurs.

Earlier, we suggested⁷ that Z-enzyme might be a dormant form of α -amylase. However, when soya beans were germinated, we found that the increase in Z-enzyme activity was not significant, compared to that which occurs in barley. It would appear, therefore, that Z-enzyme is similar to other plant α -amylases, but is normally present in extremely small quantities in the soya bean.

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SUMMARY

A method for the isolation and purification of the Z-enzyme in soya beans is described. This procedure involves the formation of the glycogen-enzyme complex.

A method for assay of activity is presented. The properties of the purified enzyme have been studied; in particular, the variation of activity and stability with temperature and pH has enabled the nature of the active sites in the enzyme to be investigated. Characterization of the activity of Z-enzyme at pH 3.6, and in the presence of a variety of reagents, has been achieved by viscometric techniques. Both linear and branched glucans are attacked by the purified enzyme. It is concluded that the properties of Z-enzyme are similar to those of other plant α -amylases.

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STUDIES ON STARCH-DEGRADING ENZYMES

PART III. THE ACTION PATTERN OF SOYA-BEAN Z-ENZYME

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INTRODUCTION

In Part II of this series¹, the purification and general properties of the Z-enzyme from soya-bean were described. We now report the first studies of the action pattern of this α -amylolytic enzyme. Linear amylose² has been used as the substrate for these investigations, as the production of maltodextrins is uncomplicated by branched products. The initial stage of amylolysis has been studied by viscometry, whilst maltodextrin formation at the "achroic limit" of the reaction has been followed by paper-chromatographic techniques.

EXPERIMENTAL

The preparations of the Z-enzyme and the substrates have been described earlier. Maltodextrins* (G_1 , G_2 , ... G_9) were isolated, by the paper-chromatographic technique described by Commerford *et al.*³, from the salivary α -amylolysis of amylose; control samples of maltodextrins were kindly donated by Professor W.J. Whelan. Qualitative separations of maltodextrins from digests were made on Whatman No. 1 chromatography paper by the multiple-descent technique, using ethyl acetate-pyridine-water (10:4:3; v/v) at 20°. Chromatograms were developed using the reagents of Trevelyan *et al.*⁴. For quantitative work on 3MM paper, a multiple-ascent method⁵, using 70% aqueous propanol, was found to give more satisfactory separations. Chromatograms using this solvent were also irrigated at 20°.

Kinetics of the initial action of Z-enzyme on amylose

The initial action of Z-enzyme on amylose was followed viscometrically⁶ at 25°. Linear amylose ($[\eta] = 400$ in 0.2M KOH; 3 mg/ml; DP , ca. 3000) was dissolved in buffer at pH 5.5 (0.01M; acetate), and the specific viscosity (η_{sp}) of the solution was determined. After the addition of enzyme (0.05 units of activity¹), η_{sp} was measured at regular intervals for 24 h. A comparable experiment was carried out at pH 7.8 (McIlvaine's buffer, 0.001M).

The hydrolysis of the amylose in 0.5M hydrochloric acid was studied similarly.

* G_1 = D-glucose; G_2 = maltose; G_3 = maltotriose; etc.

Production of maltodextrins

Amylose solution (80 ml, unbuffered, 1.3 mg/ml) was incubated with Z-enzyme (15 units of activity¹) at 35°. At intervals, aliquots (2 ml) were removed for measurement of reducing power and iodine staining. Portions (10 ml) of the digest, after being heated on a boiling-water bath for 5 min to deactivate the enzyme, were concentrated on a rotatory evaporator, and analysed by paper chromatography.

In quantitative estimations, sugars were removed from the paper by irrigation with water. Amounts were determined by hydrolysing the oligosaccharides to D-glucose (1.5N HCl for 2 h), and estimating the reducing power by alkaline ferricyanide². Control experiments showed that oligosaccharide recovery was 97–100%.

RESULTS AND DISCUSSION

The hydrolytic effect of soya-bean Z-enzyme on linear amylose was first established by Banks *et al.*⁷, when a crude enzyme preparation was found to decrease the limiting-viscosity number ($[\eta]$) of the polysaccharide. Our recent preparation and study of the properties of purified Z-enzyme have confirmed the α -amylolytic character of this enzyme¹. As with other α -amylases, hydrolysis of amylose occurs in two apparently distinct stages. First, there is a rapid decrease in the size of the amylose molecule, as shown by the fall in iodine stain, and the increase in the reducing power of the solution. This initial stage in the reaction was studied viscometrically. The second stage of the reaction begins when the "achroic limit" of the amylose solution is reached, and is characterised by a slow increase in reducing power. The apparent discontinuity in the hydrolysis reaction occurs at *ca.* 30% conversion into maltose. However, as we have stressed elsewhere¹, the "achroic limit" is an arbitrary concept, since it depends on the amylose:enzyme ratio. The production of maltodextrins at this stage in the reaction has been investigated by qualitative and quantitative paper chromatography.

Kinetics of the initial hydrolysis of amylose

The major problem here is to determine whether the hydrolysis of the α -(1→4)-glycosidic bonds is a random or non-random process. This can be determined by following the rate of substrate degradation. Ideally, number-average methods should be used to follow the change in degree of polymerization (*DP*) but, experimentally, these techniques are extremely difficult, and, furthermore, they are insensitive to limited degradation. In this work, therefore, we have used the viscometric technique, as this gives a very sensitive measure of the initial rupture of bonds in a polymer. The decrease in *DP* with time is not a true measure of degradation rate but, for either a zero- or first-order reaction, the rate is proportional⁸ to DP^{-1} . Now $[\eta] = K' (DP)^\alpha$, but, as it is more convenient to measure the change in η_{sp} with time we have evaluated DP^{-1} from the relationship⁹

$$DP^{-1} = [K'c(\eta_{sp}^{-1} + k)]^{1/\alpha},$$

where K' is a constant, c is the polymer concentration, α is the exponent in the Staudinger relation above, and k is Huggins' constant. Values of k and α were taken from the results of Banks and Greenwood¹⁰.

Vink⁹ has shown that, for a *random* hydrolytic process, the graph of DP^{-1} *versus* time is linear; whilst non-random degradation processes yield non-linear graphs.

The results of treating the data from the experiments involving the action of Z-enzyme on amylose are shown in Figure 1, curves 1 and 2. It can be seen that, for both pH 5.5 and 7.8 (*i.e.*, at optimum and adverse pH-values¹), the graph of DP^{-1} *versus* time is linear. This suggests that in the initial stages of α -amylolysis the enzymic attack is essentially a random scission of α -(1 \rightarrow 4)-glycosidic linkages. This conclusion was substantiated by the analogous results found when hydrolysis of the amylose by 0.5M hydrochloric acid — a known random process — was studied. The linear relation obtained under these conditions is shown by curve 3 in Fig. 1.

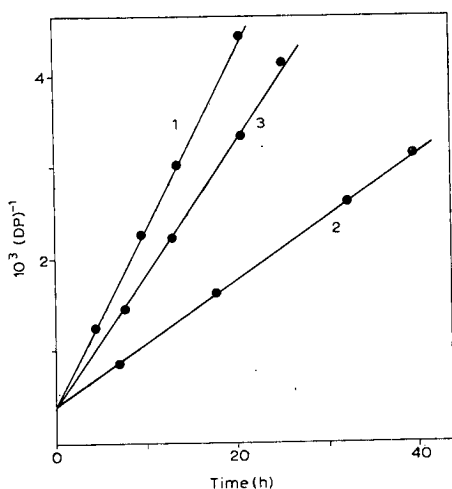


Fig. 1. Graph of DP^{-1} *versus* time for the action of soya-bean α -amylase on linear amylose: 1, pH 5.5; 2, pH 7.8; curve 3 shows the corresponding result for 0.5M hydrochloric acid.

It must be emphasised, however, that, although a large change had taken place in the value of η_{sp} by the end of these measurements, the corresponding average number of bonds broken per initial amylose molecule was only 16, and that the percentage of bonds broken during the period of investigation was therefore about 0.5.

Production of maltodextrins at the achroic point

The results of qualitative studies on the production of various maltodextrins from amylose, by Z-enzyme at the apparent achroic point (in this instance, after 33 h), are shown in Table I. It can be seen that products greater than G_6 predominate at this stage in the hydrolysis, although small quantities of the lower sugars are also present. As the hydrolysis proceeds, all of the saccharides separated (G_1 – G_9) increase

in amount, and then the higher ones begin to decrease until only trace amounts of G_5 , G_6 , and G_7 remain. Saccharide G_4 is hydrolysed extremely slowly, and is always present in the digest.

TABLE 1

PRODUCTION OF MALTODEXTRINS BY THE ACTION OF Z-ENZYME ON AMYLOSE

Maltodextrin ^a	Digest incubation time (h)				
	33	70	96	120	140
G_1	+ ^b	++	+++	++++	+++++
G_2	+	++	+++	++++	+++++
G_3	+	++	+++	++++	+++++
G_4	+	++	+++	++++	+++++
G_5	+	++	+++	++	+
G_6	++	++	+++	++	+
G_7	++	++	+++	++	+
G_8	++	++	+++	+	o
G_9	++	++	++	+	o
> G_9	++	++	+	o	o
Iodine stain ^c	Blue	Blue	Red	Achroic	Achroic

^a G_1 = D-glucose; G_2 = maltose; G_3 = maltotriose; etc.

^b The symbols (+), (++) , etc. give an estimate of the relative quantities of each of the different sugars on the chromatogram; quantities can only be compared horizontally, and *not vertically*.

^c Stain of digest, after 100-fold concentration.

The fact that maltodextrins G_8 and G_9 continue to increase in amount after the "achroic limit" indicates that long-chain substrate molecules are still present at this point. Indeed, it was found that when *concentrated* portions of the digest were stained with iodine—in contrast to the normal diluted aliquots—a blue colour was obtained. A true achroic point was not reached until much later and, at this stage, the chromatographic evidence suggests that maltodextrins greater than G_9 are not present. This observation again stresses the arbitrary nature of the term "achroic limit".

The above results suggested that some of the lower oligosaccharides differed in their susceptibility to degradation by Z-enzyme, and so the effect of the enzyme acting directly, and under comparable conditions, on G_4 , G_5 , G_6 , and G_7 was studied. Results are shown in Table II. It can be seen that G_4 and G_5 are both somewhat resistant to enzymic degradation. Furthermore, although G_6 was hydrolysed faster than G_4 or G_5 , the rate of hydrolysis of all these maltodextrins was small compared to that for G_7 . This confirms the fact that oligosaccharides differ in their susceptibility to Z-enzyme, and parallels trends we have found for other plant α -amylases¹¹.

To confirm that, at extensive degrees of enzymic hydrolysis, the process is not completely random as shown by the yields of maltodextrins, a quantitative study of these was carried out. The results are shown in Table III. In Expt. 2, the digest was prepared in the presence of 10^{-6} M sodium *p*-chloromercuribenzoate to ensure

the inhibition of any residual β -amylase molecules that might have remained undetected in our previous tests¹.

TABLE II
PRODUCTS^a OF THE ACTION OF Z-ENZYME ON MALTODEXTRINS

Substrate ^a	Digest incubation time (h)			
	19	43	90	114
G ₄	0	G ₂ +	G ₂ ++	G ₂ +++
G ₅	0	G ₂ , G ₃ +, +	G ₂ , G ₃ ++, ++	G ₂ , G ₃ ++, ++
G ₆	G ₂ , G ₃ , G ₄ +, +, +	G ₂ , G ₃ , G ₄ 2+, 2+, 2+	G ₂ , G ₃ , G ₄ 3+, 3+, 3+	G ₂ , G ₃ , G ₄ 4+, 4+, 4+
G ₇	G ₁ , G ₃ , G ₄ , G ₆ +, +, +, + G ₂ , G ₅ +, +	G ₁ , G ₃ , G ₄ , G ₆ 2+, 2+, 2+, 2+ G ₂ , G ₅ +, +	G ₁ , G ₃ , G ₄ , G ₆ 3+, 3+, 3+, 3+ G ₂ , G ₅ 2+, 2+	G ₁ , G ₃ , G ₄ , G ₆ 4+, 4+, 4+, 4+ G ₂ , G ₅ 2+, 2+

^a Symbols as in Table I.

TABLE III
YIELDS OF MALTODEXTRINS PRODUCED FROM THE ACTION OF Z-ENZYME ON AMYLOSE^a

Expt.	% by weight ^b of						Higher oligomers
	G ₁	G ₂	G ₃	G ₄	G ₅	G ₆	
I	4	13	9	7	5	21	41
2a ^c	3	9	10	6	5	17	50
2b ^c	7	13	11	7	6	31	25

^a For digest conditions, see EXPERIMENTAL.

^b Values have been quoted to the nearest integer.

^c Digest contained 10⁻⁶M sodium *p*-chloromercuribenzoate; incubation period of digest *b* was twice that of *a*.

Theory for the production of oligomers from the hydrolysis of polymers

The statistical theory of Kuhn¹² and Montroll and Simha¹³ shows that the weight fraction of *i*-mer (*W_i*) produced from the *random* scission of a polymer of *x* units in length is

$$W_i = \frac{i}{x} [2s(1-s)^{i-1} + (x-i-1)s^2(1-s)^{i-1}] \tag{1}$$

where *s* is the degree of scission. Now, the change in this yield with *s* is given by the differential

$$\frac{dW_i}{ds} = \frac{i}{x} [2(1-s)^{i-1} - 2s(i-1)(1-s)^{i-2} + 2s(x-i-1)(1-s)^{i-1} - (x-i-1)(i-1)s^2(1-s)^{i-2}].$$

Here, positive terms represent the formation of i -mer from larger oligomers, and negative terms give the degradation of i -mer to smaller fragments.

Now, as Painter¹⁴ has pointed out, *if the i -mer is resistant to further attack, but all higher oligomers may be degraded*, the final yield of i -mer is given by the integral of the positive terms in the above differential equation, *i.e.*,

$$W_i = \frac{i}{x} \int_0^1 [2(1-s)^{i-1} + 2s(x-i-1)(1-s)^{i-1}] ds$$

$$= \frac{2}{i+1} \quad (2)$$

Thus, under these conditions, the yield of i -mer is independent of the size and size distribution of the original polymer, and hence will represent the weight fraction of totally degraded polymer existing as i -mer.

Similarly, if all oligomers smaller than the i -mer are also resistant to hydrolysis, the weight fraction of $(i-j)$ -mer is¹⁴:

$$\frac{2}{(i-j+1)} \prod_{k=0}^{j-1} \frac{(i-k-1)}{(i-k+1)} \quad \begin{array}{l} i = 3, 4, 5, \text{ etc.} \\ j = 1, 2, 3, \text{ etc.} \end{array}$$

But we have shown that this relation simplifies to

$$W_{i-j} = \frac{2(i-j)}{i(i+1)} \quad \text{when } j < i \quad (3)$$

This relation gives the theoretical yields of oligomers when a polymer is degraded *completely* to oligomers of size i and smaller. However, in our experiments, the mixture of oligomers was analysed before degradation was complete, and hence, if a fraction x of total polymer has been degraded to oligomers of size i and smaller, fractional yields of oligomers can be calculated from

$$W_{i-j} = \frac{2(i-j)}{i(i+1)} \cdot x \quad (4)$$

Mechanism of the action of Z-enzyme on amylose

In Table IV, the experimental yields of oligosaccharides are compared to theoretical yields calculated on the assumption that degradation is random, but that (i) G_5 and smaller oligomers are effectively resistant, (ii) G_6 and smaller oligomers are effectively resistant, and (iii) no oligomers are effectively resistant to α -amylolysis.

In the application of Kuhn's theory^{12,13} to calculate yields for assumption (iii), a value for the degree of scission (s) was arbitrarily chosen so that the total yield of oligomers $> G_6$ approximated to that found experimentally. Now, it can be easily shown that, for any other value of s , there is a correspondingly similar theoretical distribution of the oligomer yields, *i.e.*, there is a *continuous* increase in amount up to a certain oligomer, followed by a *continuous* decrease thereafter; an experimental determination of s to demonstrate this point is therefore unnecessary.

It can be seen that our experimental yields do not follow such a pattern, and so, notwithstanding the evidence from the viscosity experiments, the α -amylolysis cannot be random at the later stages.

TABLE IV

CALCULATED YIELDS OF MALTODEXTRINS

Expt. ^a	Theoretical assumption	% weight of total						Higher oligomers
		G ₁	G ₂	G ₃	G ₄	G ₅	G ₆	
I	—	4	13	9	7	5	21	41
	(i) G ₅ stable ^b	2.5	5.1	7.6	10.1	12.7	62.0	
	(ii) G ₆ stable ^c	2.8	5.6	8.4	11.2	14.0	16.9	41.0
	(iii) Random ^d	7.6	11.0	11.9	11.6	10.5	9.1	38.3
2a	—	3	9	10	6	5	17	50
	(i) G ₅ stable ^b	2.2	4.4	6.6	8.8	11.0	67.0	
	(ii) G ₆ stable ^c	2.4	4.8	7.1	9.5	11.9	14.3	50.0
	(iii) Random ^d	5.1	7.8	9.1	9.4	9.1	8.5	49.0
2b	—	7	13	11	7	6	31	25
	(i) G ₅ stable ^b	3.0	6.0	9.0	12.0	15.0	55	
	(ii) G ₆ stable ^c	3.6	7.1	10.7	14.3	17.9	21.4	25
	(iii) Random ^d	11.1	14.8	14.8	13.2	11.0	8.8	26.3

^a See Table III.

^b Calculated from equation 4 with $i = 5$; $j = 0, 1, \dots, 4$; $x = 0.38$ for Expt. I; $x = 0.33$ for Expt. 2a; $x = 0.45$ for Expt. 2b.

^c Calculated from equation 4 with $i = 6$; $j = 0, 1, \dots, 5$; $x = 0.59$ for Expt. I; $x = 0.50$ for Expt. 2a; $x = 0.75$ for Expt. 2b.

^d Calculated from equation 1 with a value of s chosen so that the yield of G₇ and $> G_7$ was comparable to that found experimentally.

Again, when the inherent stability of certain maltodextrins is assumed, better agreement between experiment and theory occurs for (ii) than (i). But, in both cases, more G₆ and G₂, and less G₄ and G₅ are found experimentally than are predicted, confirming that the α -amylolysis cannot be random.

The presence of excess of G₂ and G₆ may be explained by postulating that, when the enzyme attacks an oligosaccharide, it is unable to attack readily (a) the bond nearest the reducing end-group, and (b) the five bonds nearest the non-reducing end-group.

Now with G₁₂, for example, if (a) holds, the yields of products formed would be G₁ = 26.4%; G₂ = 73.6%. For (b), the final yields would be: G₁ = 8.3%; G₂ = 8.3%; G₃ = 8.3%; G₄ = 8.3%; G₅ = 8.3%; G₆ = 58.5%. For (a) and (b) to hold together, the yields from G₁₂ would be: G₁ = 0; G₂ = 10.5%; G₃ = 9.2%; G₄ = 8.8%; G₅ = 8.3%; G₆ = 41.6%; G₇ = 21.7%.

Although none of these schemes accounts adequately for the observed yields,

the last one is more satisfactory as it shows the tendency to form more G_2 and G_6 , and less G_4 and G_5 . It is perhaps reasonable, therefore, to consider that an intermediate scheme may explain the results, *i.e.*, that some bonds in a linear amylose molecule are *more resistant* to α -amylolytic attack than others. It seems likely that these particular bonds are the one adjacent to the reducing group, and the five bonds adjacent to the non-reducing end. This hypothesis is similar to that proposed by Bird and Hopkins¹⁵.

Elsewhere¹⁶, we have developed this hypothesis, and shown that it will explain the preferential non-random degradation of oligosaccharides by α -amylase.

The apparent *random* degradation of amylose in the initial stages is not inconsistent with this scheme in which the enzyme will not hydrolyse the bonds near the ends of molecules as readily as others; in a molecule of degree of polymerization 3000, there will be only 6 bonds which are resistant to attack, leaving 2993 bonds which may be attacked *randomly*. Initially, therefore, the degradation will appear to be a random process but, as α -amylolysis proceeds, the proportion of resistant bonds increases and the rate of enzymic attack will decrease. In the final stage, when only G_8 and $< G_8$ are present, there are few non-resistant bonds in the substrate molecules and the reaction is very slow and non-random.

The two apparent stages in the α -amylolysis of amylose may thus merely be a result of the difference in affinity of the enzyme for large and small substrate molecules.

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SUMMARY

The action pattern of soya-bean Z-enzyme on linear amylose has been investigated. The kinetics of the initial stages of α -amylolysis have been studied by the use of viscometric techniques, and the process is shown to be an apparently random one. Paper-chromatographic studies of the production of maltodextrins at the "achroic limit" indicated, however, that, at this stage, the attack is non-random. This has been confirmed by (i) studying the action of the enzyme on individual maltodextrins, and (ii) measuring quantitatively the yields of dextrins after the "achroic limit". The latter experimental yields have been compared with those calculated from various theoretical models for the α -amylolytic action. A hypothesis has been developed to account for the observations made at various stages of the α -amylolysis.

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The α -Amylase from Broad Beans

Purification and Properties¹

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A method for the isolation and purification of the α -amylase in the broad bean (*Vicia faba*) is described. This procedure involves the formation of the glycogen- α -amylase complex. The properties of the purified enzyme were studied, and in particular, the variation of activity with pH enabled the nature of the active sites in the enzyme to be investigated. Characterization of the activity of the enzyme at pH 3.6 and in the presence of a variety of reagents was achieved by viscometric techniques. Characteristic α -amylolytic degradation patterns for the action of the enzyme on various substrates were obtained.

Several carbohydrases have been reported to be present in the broad bean (*Vicia faba*, Linn), and detailed investigations of the phosphorylase and Q-enzyme (1, 2), and the R-enzyme (3-5) have been made. In some of this work, there was evidence for the presence of an enzyme having α -amylolytic activity; however, although Hobson and co-workers (1) found an optimum pH of 6.1-6.3, this enzyme has not been further characterized.

As part of a general study of plant α -amylases, we now describe a convenient method for the isolation and purification of broad bean α -amylase. A detailed study of its properties has also been made.

MATERIALS AND METHODS

Analysis

Routine determinations of protein concentration were made from absorption measurements at 280 m μ after an initial calibration by micro-Kjeldahl estimations ($OD_{280}^{1\%} = 1.0$ for 0.1% w/w solution). Concentrations of polysaccharide solu-

tions were determined by hydrolysis followed by titration of the liberated glucose with alkaline ferrieyanide (6). β -Amylolysis limits, $[\beta]$, were carried out with crystalline sweet potato β -amylase (Worthington Biochemical Corporation) as described earlier (6). Measurements of the limiting viscosity number, $[\eta]$, were made in 0.2 M potassium hydroxide at 25°C. Unless otherwise stated, enzymic digests were carried out at 35°C, and the pH was controlled at 5.5 with acetate buffer (0.2 M). Phosphate estimations were made by Allen's method (7), and the activity of the R-enzyme was measured as described by Hobson and co-workers (3).

Substrates

Amylose and amylopectin were prepared by the fractionation of potato starch (6). Starch was dispersed in boiling water with vigorous stirring in a nitrogen-atmosphere for 1 hour to form a 0.5% solution. After cooling to 60°C, thymol (1 gm/1 liter) was added. The amylose-thymol complex, formed on standing for 48 hours at room temperature, was removed by high-speed centrifugation (Sharples supercentrifuge). The product was then re-dispersed and re-crystallized as the butan-1-ol complex to yield amylose with $[\eta] = 500$ and $[\beta] = 80$. Amylopectin was obtained by freeze-drying the supernatant fluid from the thymol complex after removal of thymol by extraction with ether. The limiting viscosity number of the product was 180 and the β -limit was 56. Linear amylose was obtained by leaching methanol-pretreated potato

¹ Part 4 in the series, "Studies on Starch-Degrading Enzymes"; part 3 is *Carbohydrate Res.*, in press. This work was supported by a grant from the Corn Industries Research Foundation, Inc., Washington, D. C., and a postgraduate studentship to E.A.M. from the Department of Scientific and Industrial Research, London.

TABLE I
 α -AMYLOLYTIC ACTIVITY OF PROTEINS OBTAINED FROM BROAD BEAN BY ACETONE
 FRACTIONATION AT -5°C

Initial fractionation							
% Acetone (v/v)	0-30	30-35	35-40	45-45	45-50	50-55	55-60
Fraction	1	2	3	4	5	6	7
Activity ^a	0.86	1.00	1.33	1.43	2.25	3.30	0.42

Second fractionation							
% Acetone (v/v)	30-35	35-40	40-45	45-50	50-55	55-60	60-70
Fraction	A	B	C	D	E	F	G
Activity ^a	0.96	3.30	6.4	10.6	24.0	12.0	3.3

^a Specific activity of enzyme: see text.

starch granules at 60°C ; a 0.5% aqueous suspension was slowly stirred in a nitrogen atmosphere. After cooling to room temperature, the swollen granules were removed by centrifugation and the amylose was precipitated from the supernatant fluid by the addition of butan-1-ol. After recrystallization with butan-1-ol the amylose was found to have a β -limit of 100% and a limiting viscosity number of 260.

Glycogen was extracted from rabbit livers with cold trichloroacetic acid (8), and purified by repeated precipitation with ethanol from aqueous solution. The β -limit of the product was 45%.

Amylopectin- and glycogen- β -limit dextrans were obtained by digesting the substrate (0.5% concentration) in acetate buffer at pH 4.6, with the crystalline β -amylase. The amount of enzyme was such that digestion was complete within 3 hours. The digests were then left for 24 hours before the enzyme was deactivated by heating the digest on a boiling water-bath for 3 minutes. Liberated maltose was removed by dialysis against repeated changes of distilled water until the dialyzate possessed no reducing power, and the dextrin was then isolated by freeze-drying.

α -Amylase Activity

A modification of the procedure devised by Briggs (9) was employed. In this method, activity is expressed as the reciprocal of the time taken to decrease a dextrin-iodine stain by a standard amount. [Briggs (9) has compared this with classical methods of assay.] The substrate used was amylopectin β -limit dextrin, and the "time reference point" in the method (9) was taken as the time when the corrected absorption value, A.V., had fallen to 2.00 colorimeter units.

Digests were prepared as follows: buffer (1 ml; pH 5.5), amylopectin β -limit dextrin solution (2 ml; 12 mg), enzyme solution, and water to give a

total of 8 ml. Portions of the digest (2 ml) were removed at intervals and stained with iodine (1 ml; 0.2% in 2% KI) in the presence of hydrochloric acid (0.2 ml; 5 M) in a total of 50 ml. The A.V. was then measured at 540 m μ in an EEL-colorimeter. Specific activities were expressed as iodine dextrin color units (9) per milligram of protein per milliliter of digest. The standard graph, from which subsequent α -amylolytic activities were calculated, was constructed by measuring the effect of salivary α -amylase on the β -limit dextrin.

Enzyme Preparation

"Champion Long Pod" broad beans from Dobbie and Sons, Edinburgh, were used. In the method, concentrations are quoted as "initial concentrations."

a. *Initial extraction and fractionation.* Dry, defatted broad bean flour (100 gm) was shaken with calcium chloride solution (500 ml; 0.2%) containing *n*-octanol (0.2 ml) for 2 hours at 18°C . The suspension was centrifuged at 900g to yield a clear supernatant fluid (35 mg of protein per milliliter; specific activity of α -amylase = 0.54). After cooling, the proteins were fractionated by the addition of cold acetone at -5°C . The resultant fractions obtained by centrifugation (at -5°C ; 1100g) were air-dried and dissolved in water at 2° . Their activities were as shown in Table I.

b. *Refractionation with acetone.* Fractions 5 and 6 (Table I) were combined and refractionated with acetone at -5°C . The specific activities of the resultant subfractions are also shown in Table I.

c. *Glycogen-complex formation* (10). Ethanol (at 2°C) was slowly added with stirring to fractions E + F (Table I) to 40% (v/v). After 15 minutes, any precipitated protein was removed by centrifugation (1100g) at 2°C . Phosphate buffer (0.5 ml/10 ml enzyme solution; pH 8.0; 0.2 M) and glycogen (2%; 0.2 ml/10 ml of enzyme solution) were then

added to the enzyme in 40% (v/v) ethanol. The suspension was stirred for 5 minutes and then centrifuged at 1100*g*. The precipitated complex was dissolved in water, and the resultant solution was maintained at 18°C for 6 hours to hydrolyze the glycogen. It was then dialyzed against calcium acetate (0.2%) at 2°C for 12 hours and was stored at this temperature. This α -amylase solution had a specific activity of 360 iodine dextrin colour units per milligram of protein per milliliter of digest, and was stable for several weeks.

Purity of the Enzyme

To test for the presence of contaminating carbohydrases, the following digests were prepared and incubated at 35°C:

(a) Laminarin (1 mg in 0.1 ml) and enzyme (0.1 ml); maltose (1 mg in 0.1 ml) and enzyme (0.1 ml); cellobiose (1 mg in 0.1 ml) and enzyme (0.1 ml). Portions of each digest were removed at intervals for chromatographic examination. No glucose was detected in any of the digests.

(b) Maltotriose (1 mg in 0.1 ml) and enzyme (0.1 ml). On chromatographic examination of aliquots, traces of glucose and maltose were present after 10 hours of incubation, but even after 48 hours, no sugars larger than maltotriose were found.

(c) Glucose-6-phosphate (5 mg in 1 ml), enzyme (0.2 ml), acetate buffer (0.5 ml; 0.2 *M*; pH 4.6), and water (2 ml). After 24 hours, the presence of free phosphate groups was tested by Allen's method (7). No phosphate was detected.

(d) Amylose (10 ml; 1 mg/1 ml), acetate buffer (1 ml; 0.2 *M*; pH 5.5), disodium hydrogen phosphate (1.5 ml; 23 mg/1 ml), and enzyme (0.2 ml). Portions of the digest were removed at intervals for free phosphate determinations. After 48 hours, there was no reduction in the amount of free phosphate in the digest.

Effect of Temperature and pH

To obtain the temperature/activity dependence for the enzyme, digests at pH 5.5 were preincubated to temperature equilibrium before enzyme was added. In the stability measurements at pH 5.5, the enzyme solution and buffer were kept at the appropriate temperature for 1 hour before cooling to 35°C and adding the dextrin. Resultant activities were then compared with those obtained at 35°C without prior incubation.

The pH-dependence of activity at 35°C was obtained by using disodium hydrogen phosphate-acetic acid buffers; McIlvaine's standard buffer solutions were found to completely inactivate the enzyme. The effect of pH on stability was determined by maintaining the enzyme and buffer at 20°C for 75 minutes. Digests were then brought to

5.6 and incubated with dextrin at 35°C, and the activity was determined.

To study the effect of pH 3.6 in more detail, the following digests were prepared:

a. Buffer (2 ml; pH 5.5) + enzyme (0.2 ml) + amylose (10 ml; 2 mg/1 ml).

b. Buffer (2 ml; pH 3.6) + enzyme (0.2 ml) + amylose (10 ml; 2 mg/1 ml).

c. Buffer (2 ml; pH 3.6) + enzyme (0.2 ml); incubated 2 hours at 20°C before amylose (10 ml; 2 mg/1 ml) was added.

After incubation at 35°C for 5 hours, the amylose present in each digest was precipitated with excess butanol and washed with water saturated with butanol, and the $[\eta]$ -value was then measured.

Effect of Various Reagents on Activity

Enzyme and reagent were kept at 20°C for 2 hours before the addition of amylopectin β -limit dextrin, and the activity was determined in the usual manner. Further, the effect of EDTA and trypsin on α -amylolytic activity was studied by preparing digests *d-f* by adding to 10-ml portions of enzyme and buffer at pH 5.5: for *d*, EDTA (1 ml; 10^{-1} *M*); for *e*, EDTA (1 ml; 10^{-1} *M*) and trypsin (1 ml; 6 mg per milliliter); for *f*, trypsin (1 ml; 6 mg per milliliter). A control digest *g* was also prepared. The mixtures were kept at 20°C for 12 hours, and amylose solution (10 ml; 2 mg per milliliter) was then added to each. After a further 48 hours, excess butanol was added. There was no amylose complex in digests *f* and *g*, but the $[\eta]$ -values for the residual polysaccharide in digests *d* and *e* were determined.

Digests *h-m* were prepared to study the effect of Ca^{++} on this system by adding to 2.5-ml portions of enzyme and buffer at pH 5.5: for *h* and *i*, EDTA (1 ml; 10^{-1} *M*); for *j* and *k*, trypsin (0.2 ml; 6 mg per milliliter); for *l* and *m*, EDTA (1 ml; 10^{-1} *M*) and trypsin (0.2 ml; 6 mg per milliliter). A control digest *n* was also prepared. The total volume was then adjusted to 5 ml and each mixture was incubated for 12 hours at 20°C when calcium chloride (50 mg) was added to digests *i*, *k*, and *m*. Equal volumes (4 ml) of the same amylopectin β -limit dextrin solution (1.5 mg per milliliter) were then added to all the digests, and activities were determined as usual.

Modification of the Enzyme

The broad bean α -amylase was coupled with diazobenzene sulfonic acid and acetylated as described by Fraenkel-Conrat (11).

RESULTS AND DISCUSSION

Purification of broad bean α -amylase. It must be stressed first that the amount of

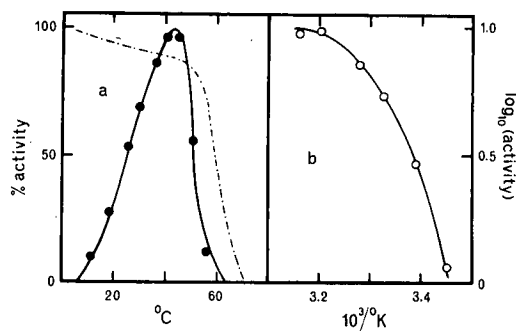


FIG. 1. (a) Effect of temperature, $^{\circ}\text{C}$, on the activity (—●—) and stability (---) of broad bean α -amylase. (b) Arrhenius plot of the temperature dependence of the activity.

α -amylase in the broad bean is extremely small, and it is barely detectable by classical assay methods.

In view of our earlier investigations of plant α -amylases (12, 13), fractionation of the proteins in an aqueous extract of the broad bean was made with acetone at -5°C . After two such fractionations, the α -amylase was purified by the formation of a glycogen complex (cf. Ref. 10). The reaction is extremely specific for this enzyme (10). This procedure yielded a product in which the α -amylolytic specific activity was 700 times greater than in the original extract. Maltase, laminarase, and cellobiase were absent from the purified product as shown by digestion with the appropriate substrate followed by chromatographic analysis. Incubation with maltotriose showed the presence of only traces of glucose and maltose, i.e., the enzyme attacked this substrate slowly, and the absence of higher sugars showed that D-enzyme was not a contaminant. The absence of phosphatase was shown by the inability of the enzyme to hydrolyze glucose-6-phosphate, and since a digest containing amylose, phosphate, and enzyme showed no change in phosphate content, phosphorylase was absent. Further, there was no shift in the absorption spectra of the iodine complex as the α -amylolysis proceeded, showing that no R-enzyme was present. A digest containing enzyme, which had been preincubated at pH 3.6 for 2 hours at room temperature, and soluble starch solution (0.6%) was maintained at 35°C for 24 hours. No change in the reducing power of the digest

was found, indicating the absence of β -amylase.

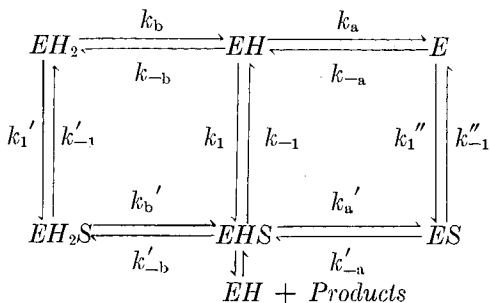
Effect of temperature and pH on activity and stability. Figure 1a shows that the enzyme exhibits its maximum activity at about 45°C . Stability experiments indicated that some 90% of the activity is retained after 1 hour's incubation at this temperature, while there is a very rapid decrease in stability at temperatures between 55° and 65°C . The temperature dependence of the activity was used to estimate the apparent heat of activation of the enzyme. Figure 1b shows the appropriate Arrhenius plot; the heat of activation varies from 14 kcal at 9°C , to 5 kcal at 25°C and is apparently zero at 45°C .

The loss of enzyme activity which occurred in phosphate-citrate buffers must be attributed to the removal of calcium ions from the enzyme (see later). It is to be noted that the action of phosphate and citrate ions separately did not affect the activity; their combined action was necessary to cause marked deactivation.

The effect of pH on the activity of the enzyme in phosphate-acetate buffer is shown in Fig. 2a, where the ratio, (activity at a given pH, V_H)/(maximum activity at optimum pH, V_{\max}), is plotted against the pH. The pH of maximum activity is 5.6, and the shape of this curve was independent of the concentration of substrate.

Figure 2b shows the stability of the enzyme at various pH values; a very rapid irreversible denaturation occurs at pH's lower than 5.6.

Assuming that the enzyme has two reactive groupings, the following hypothetical scheme (14-17) was used to analyze the pH-activity curve:



where E = enzyme, H = proton, S = substrate, and k = velocity constants as indi-

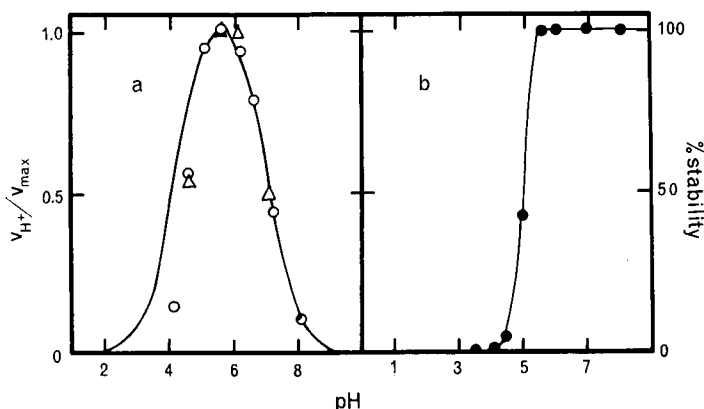


FIG. 2. (a) Effect of pH on the activity (V_{H^+}/V_{max}) of broad bean α -amylase. (○) Substrate concentration = 0.7 mg/ml; (Δ) substrate concentration = 2.8 mg/ml. Solid line represents the theoretical curve discussed in the text. (b) Effect of pH on the stability of broad bean α -amylase.

TABLE II
VISCOSITIES OF POLYMER PRODUCTS AFTER
DIGESTION OF AMYLOSE ($[\eta] = 500$)

Digest ^a	Conditions ^a	$[\eta]$ of amylose product
a	pH 5.5, control	90
b	pH 3.6, directly	430
c	pH 3.6, preincubation	500
d	EDTA	470
e	EDTA + trypsin	500
f	Trypsin	0
g	No EDTA or trypsin	0

^a See *Materials and Methods*.

cated. Ionization constants of the enzyme (K_a , K_b) and enzyme-substrate complex (K'_a , K'_b) are defined as

$$K_a = k_a/k_{-a}, K_b = k_b/k_{-b}$$

$$K'_a = k'_a/k'_{-a}, K'_b = k'_b/k'_{-b}.$$

If it is assumed that the form EHS is the only complex capable of reacting to yield products, and the ionization of the two groups is unaffected by substrate binding, then

$$\frac{V_H}{V_{max}} = \frac{1 + (2K_a/K_b)^{\frac{1}{2}}}{1 + K_a/[H] + [H]/K_b}$$

(as $K_a = K'_a$; $K_b = K'_b$),

i.e., V_H/V_{max} is independent of substrate concentration. Our experimental data showed such an independence, and hence this function was used to calculate K_a and K_b .

A value for pK_a of 7.1 was obtained. The

instability of the enzyme at low pH-values made the determination of pK_b less accurate; however, when $[H] = K_b$, $V_H/V_{max} = 0.5$, inspection of the pH-activity curve indicates a maximum value of $pK_b \approx 4.5$. Again the pH of maximum activity is given by $[H] = (K_a/K_b)^{\frac{1}{2}}$, and hence for a pH of 5.6 and $pK_a = 7.1$ the corresponding value of pK_b is about 4.1.

The solid line in Fig. 2a represents the function above (i.e., V_H/V_{max}) calculated for values of $pK_a = 7.1$ and $pK_b = 4.1$; experimental values for pH < 5.6 naturally lie off this curve.

The apparent activity of the enzyme at pH 3.6 was low. This pH is important in the study of Z-enzyme (12), and so its effect on broad bean α -amylase was investigated in more detail by following the limiting viscosity number, $[\eta]$, of the polymer product in the digest. Table II shows that the enzyme was completely inhibited by 2 hour's incubation at pH 3.6 (digest c).

TABLE III
EFFECT OF CALCIUM IONS ON ACTIVITY

Digest ^a	Conditions ^a	% of original activity
h	EDTA	3
i	EDTA + Ca^{++}	30
j	Trypsin	90
k	Trypsin + Ca^{++}	88
l	EDTA + trypsin	0
m	EDTA + trypsin + Ca^{++}	0
n	No EDTA or trypsin	100

^a See *Materials and Methods*.

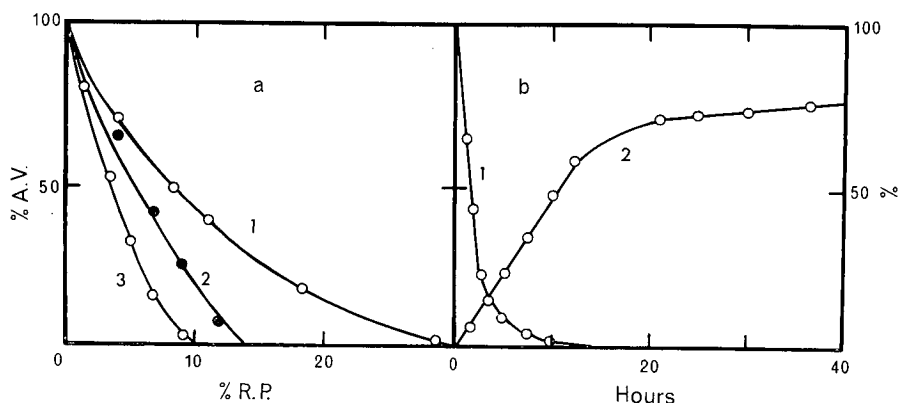


FIG. 3. (a) Graph of percentage of original absorption value of iodine complex, A.V., versus apparent percentage conversion into maltose, R.P., for (1) amylose, (2) amylopectin, and (3) amylopectin β -limit dextrin. (b) Graph of action of broad bean α -amylase on amylose as function of time, showing (1) percentage of original A.V. and (2) apparent percentage conversion into maltose.

Nature of the active centers. The nature of the ionizing groups in the enzyme concerned with the breakdown of the enzyme-substrate complex may be inferred from the pK values determined above. The group of $pK_a = 7.1$ is most probably an imidazolium ring (as in histidine), and the group of $pK_b \approx 4.1$ is likely to be a carboxyl group (as in aspartic or glutamic acid) (18).

In order to investigate the nature of the group with $pK = 7.1$, digests were prepared incorporating $3 \times 10^{-6} M$ iodine, and $3 \times 10^{-5} M$ sodium *p*-chloromercuribenzoate. The iodine caused complete inhibition of α -amylolytic activity, but the *p*-chloromercuribenzoate had no effect. Destruction of enzyme activity by iodine would suggest that phenol, imidazolium, or sulfhydryl groups are essential constituents for the active centers. However, the lack of effect of the *p*-chloromercuribenzoate indicates that sulfhydryl groups are unnecessary. Thus phenol or imidazolium groups, or both, seem to form part of the active center. This was confirmed when there was a 70% reduction of activity after coupling the enzyme with diazobenzene sulfonic acid. Acetylation of the protein with acetic anhydride lowered the enzymic activity by 45%. This reagent is fairly specific for amino groups, but may also attack sulfhydryl and phenol groups. However, insufficient enzyme was available for a quan-

TABLE IV
EFFECT OF BROAD BEAN α -AMYLASE ON GLYCOGEN

Digest period (hours)	% Conversion into maltose	
	Glycogen	Glycogen β -limit dextrin
0	0	0
5	4	0
12	8	2
30	10	2
48	11	3

titative estimation of the extent and position of acylation, and hence it is not known whether amino groups are important for the amylase activity, or whether the reduction in activity is due to partial acetylation of some other grouping.

Effect of various reagents on activity. Investigations showed that potassium cyanide and tryptophane in the range of 10^{-3} – $10^{-6} M$ had no effect on the activity, and over this concentration range neither sodium chloride nor calcium chloride activated the enzyme. Mercuric chloride at 10^{-3} – $10^{-4} M$ caused complete inhibition, but at $10^{-5} M$ there was 85%, and at $10^{-6} M$ 52% inhibition. Ammonium molybdate ($10^{-4} M$) had little effect (some 5% inhibition).

The importance of the calcium ion to α -amylase activity has been extensively investigated by Fischer and his colleagues (19). In our experiments, we have studied

the effect of the removal of calcium from the enzyme by EDTA alone, and in the presence of trypsin, by measuring changes in the $[\eta]$ -value of amylose under these conditions. Table II shows that the combined action of EDTA and the protease, trypsin, caused complete loss of α -amylolytic activity (digest *e*). This suggests that the broad bean α -amylase also requires calcium ions for its activity. EDTA alone (digest *d*) does not cause complete inhibition, while trypsin acting alone (digest *f*) has no effect on the activity. The effect of the subsequent addition of calcium ions showed that activity in the presence of EDTA could be partly restored (Table III, digest *i*), but that the concurrent effect of EDTA and trypsin was irreversible (Table III, digest *m*). Thus it appears that the enzyme may be rendered susceptible to protease attack in the absence of bound calcium.

Action on various substrates. Characteristic α -amylolytic action patterns of the broad bean enzyme on amylose, amylopectin, and amylopectin β -limit dextrin are shown in Fig. 3a as the percentage loss in iodine-staining ability as a function of the reducing power. Apparent "achroic limits" occur at about 30% conversion into maltose for the amylose; at about 14% conversion for amylopectin; and at about 10% conversion for the limit dextrin. However, as has been stressed elsewhere (12, 13), the "achroic limit" is an arbitrary concept.

As with other α -amylases, there were apparently two distinct stages in the hydrolysis of amylose as shown in Fig. 3b. First, there was a rapid decrease in size of the amylose molecule as shown by the fall in iodine-staining ability and the increase in reducing power. This is then followed by a slow increase in reducing power at the "achroic limit."

α -Amylolytic of glycogen and its β -limit dextrin was followed by reducing-power measurements as in Table IV. It can be seen that glycogen β -limit dextrin is attacked

some 3-4 times slower than glycogen itself, indicating that exterior chains in the parent molecule are more readily hydrolyzed than internal ones.

The action pattern of the enzyme is considered in detail elsewhere (20).

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Action Pattern of Broad Bean α -Amylase¹

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A study was made of the mode of action of broad bean α -amylase on linear amylose. Viscometric techniques were used to show that the initial hydrolysis appears to be random.

The products of the degradation of amylose at the achroic limit, and also of the amylolysis of individual small oligosaccharides, were investigated by paper chromatography. The results indicate that the attack is nonrandom in the later stages.

Quantitative determinations of the proportions of maltodextrins produced after the achroic limit confirmed the nonrandom nature of the enzyme action. Yields of dextrins to be expected at various stages of the degradation were calculated for different theories of amylase action and compared with the experimentally determined values. A reaction scheme has been proposed to account for the observations.

The previous part of this series (1) described the purification and characterization of the general properties of the α -amylase from the broad bean (*Vicia faba*, Linn.). Here we have studied the action pattern of this enzyme. Linear amylose (2) has been used as the substrate since its hydrolysis will occur without the formation of branched products. As was shown previously (1), the α -amylolytic degradation of amylose apparently takes place in two characteristic stages. In this work we used (i) the viscometric method to study the kinetics of the first stage of the reaction, and then (ii) paper-chromatographic techniques to analyze the maltodextrins produced at the "achroic limit."

EXPERIMENTAL

The methods of analysis and the preparation of purified broad bean α -amylase and the linear amylose were described earlier (1). Maltodextrins (G_1 , G_2 , G_3 , ... G_9)² were isolated by paper

chromatography (3) from the salivary α -amylolysis of amylose; control samples were kindly donated by Professor W. J. Whelan. Qualitative separations of maltodextrins from digests were made on Whatman No. 1 chromatography paper by the multiple-descent technique; ethyl acetate-pyridine-water (10:4:3; v/v) was used as solvent at 20°C. Quantitative separations were made on Whatman 3MM paper with the multiple-ascent technique and 70% aqueous propanol (4) as solvent at 20°C.

Kinetics of initial hydrolysis. The kinetics of the initial hydrolysis of the amylose were followed viscometrically (5) at 25°C. The specific viscosity, η_{sp} , of a solution of amylose in 0.01 *M* acetate buffer at pH 5.5 [20 ml; amylose (DP \approx 3000); 1.5 mg per milliliter] was determined. After the addition of enzyme [0.05 ml; 0.05 unit of activity (1)], η_{sp} was determined at regular intervals for 40 hours.

The hydrolysis of the amylose in 0.5 *M* hydrochloric acid was determined similarly.

Production of maltodextrins. Amylose solution (74 ml; unbuffered; 3 mg per milliliter) was incubated with broad bean α -amylase [15 units of activity (1)] at 35°C. At intervals, 5-ml portions were removed, heated on a boiling water-bath for 5 minutes to deactivate the enzyme, and then concentrated to small volume on a rotatory evaporator. The samples were then analyzed by paper chromatography.

In quantitative estimations, sugars were removed from the paper by irrigation with water. Estimations of concentrations were made by

¹ Part 5 in the series, "Studies on Starch-Degrading Enzymes"; part 4 appears in this issue of *Arch. Biochem. Biophys.* This work was supported by a grant from the Corn Industries Research Foundation, Inc., Washington, D. C., and a postgraduate studentship to E.A.M. from the Department of Scientific and Industrial Research.

² G_1 = glucose; G_2 = maltose; G_3 = maltotriose; etc.

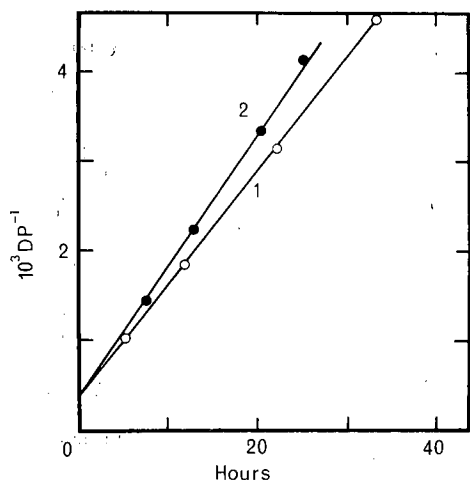


FIG. 1. Graph of DP^{-1} versus time for the amylose sample under the action of (1) broad bean α -amylase, and (2) 0.5 *M* hydrochloric acid.

hydrolyzing the oligosaccharide samples (except maltose) to glucose (1.5 *M* hydrochloric acid for 2 hours) and measuring the reducing power by alkaline ferricyanide (2). Maltose was determined directly by this method (2). (Oligosaccharide recovery was shown to be 97–100%.)

RESULTS AND DISCUSSION

The first stage in the α -amylolysis of amylose is characterized by a rapid decrease in molecular size as shown by loss of iodine-staining ability and a corresponding increase in reducing power (cf. Fig. 3 in Ref. 1). The problem here is to determine whether this hydrolysis of the α -1:4-glycosidic bonds is occurring in a random or nonrandom manner. Such a depolymerization process can be followed ideally by the use of number-average methods which count the number of molecules in the system. Unfortunately, such methods are difficult to carry out experimentally and are also insensitive to limited degradation. Weight average techniques therefore have to be used, and of these, viscosity is the most sensitive for measuring the initial rupture of bonds in the polymer. Viscosity measurements can be related then to the degree of polymerization, DP , of the polymer by the well-known Mark-Houwink equation that $[\eta] = K'(DP)^\alpha$, where K' and α are constants for a given polymer-solvent system.

For a zero- or first-order degradation

process, the rate is proportional to DP^{-1} . We have evaluated this function from measurements of the specific viscosity, η_{sp} , by using the theoretical relation of Vink (6), i.e.,

$$DP^{-1} = [K'c(\eta_{sp}^{-1} + k)]^{\alpha^{-1}}.$$

Here, c is the polymer concentration, and k is Huggin's constant. Values of k and α were taken from results of Banks and Greenwood (7) for the amylose-water system.

Vink (6) has presented a theoretical background to the study of the degradation of linear polymers, and has shown that for random degradation the graph of DP^{-1} versus time is linear.

Figure 1 shows the results of treating the data from the action of the broad bean α -amylase on the amylose sample. It can be seen that the graph of DP^{-1} versus time is linear. This indicates that the enzymic hydrolysis is essentially a process involving the random scission of α -1:4-glycosidic linkages in the initial stages. The analogous results obtained when the amylose was hydrolyzed by 0.5 *M* hydrochloric acid confirm this conclusion (curve 2, Fig. 1).

Although a very large change in the value of η_{sp} had occurred by the end of these experiments, the corresponding average number of bonds broken per initial molecule was only about 20, and the percentage of bonds broken during the period of investigation was therefore about 0.7.

Production of maltodextrins. Table I shows the results of qualitative studies of the production of the various maltodextrins from amylose by the α -amylase at the achroic point (in this experiment, 22 hours). At this stage in the α -amylolysis, maltodextrins greater than G_6 predominate, although small quantities of the lower sugars are also present. As the hydrolysis proceeds, all the maltodextrins (G_1 – G_8) increase in amount, and then the higher ones begin to decrease until there are only traces of G_7 and G_8 .

The arbitrary nature of the "achroic limit" is shown by the increase in G_8 and $>G_8$ after this point; long-chain material must still be present. In fact, when the digest was concentrated some 100-fold, a blue iodine color was obtained. The system was truly achroic only when there were no maltodextrins greater than G_7 present.

TABLE I
PRODUCTION OF MALTODEXTRINS BY THE ACTION OF BROAD BEAN α -AMYLASE ON AMYLOSE^a

Time of examination (hours):	22	32	45	58	104	140
G ₁ ^b	+	+	++	+++	++++	+++++
G ₂	+	++	+++	+++	++++	+++++
G ₃	+	++	+++	+++	++++	+++++
G ₄	+	++	++	++	+++	++++
G ₅	+	++	++	++	++	++
G ₆	+	++	+++	+++	+++++	++
G ₇	+++	++++	++++	++++	+++	+
G ₈	+++	+++++	++++	+++	+	0
>G ₈	++++	+++++	++++	+++	+	0
Iodine stain ^c	Purple	Red	Brown	Brown	Light brown	Achroic

^a The symbols (+), (++) , etc., give an estimate of the relative quantities of the different sugars on the chromatogram. The quantities can be compared only horizontally and *not vertically*.

^b G₁ = glucose; G₂ = maltose; G₃ = maltotriose, etc.

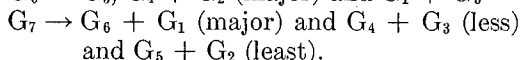
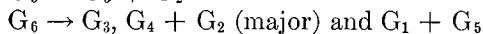
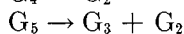
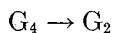
^c Stain of digest concentrated 100-fold.

TABLE II
YIELDS OF MALTODEXTRINS PRODUCED FROM THE ACTION OF BROAD BEAN α -AMYLASE ON AMYLOSE

Expt. ^a	% by weight of						Higher sugars
	G ₁	G ₂	G ₃	G ₄	G ₅	G ₆	
1a	1.8	8.5	9.6	6.2	5.5	14.3	54.0
1b	1.9	8.1	9.3	6.3	5.8	13.9	54.5
2	4.7	13.2	10.7	7.3	6.7	27.8	29.5

^a Expts. 1a and 1b are replicate determinations. The incubation period of expt. 1 was 58 hours, and that of exp. 2 was 140 hours (compare with Table I).

In order to investigate whether some of the lower maltodextrins varied in their susceptibility to the broad bean α -amylase, digests were prepared in which the α -amylase acted separately on the oligosaccharides G₄, G₅, G₆, and G₇. The rate of hydrolysis of G₄, G₅, and G₆ was found to be small compared to that for G₇. Furthermore the products of hydrolysis were:



This indicated that the maltodextrins vary

in their susceptibility to the broad bean α -amylase.

To confirm the nonrandom nature of the maltodextrin production, yields were determined by quantitative paper chromatography. Table II shows the results of these experiments. Estimations were reproducible to about 5%.

Theory of α -amylolysis. The qualitative work on the oligosaccharides indicated that G₅ and G₆ were hydrolyzed very much more slowly than G₇. The possibility that these oligomers were relatively stable to α -amylolysis was therefore considered, and the theoretical yields to be expected on this basis were calculated.

During random degradation, all bonds in the polymer have an equal probability of being broken, i.e., the product is as likely to be an n -mer as it is to be an $(n - i)$ -mer. Further, if the n -mer and smaller oligomers are resistant to attack, eventually all the larger molecules will be degraded to n -mers and smaller, and since the probability of obtaining an n -mer is the same as that of obtaining an $(n - i)$ -mer, *equal numbers* of molecules of size $n, n - 1, n - 2, \dots, 2, 1$ will be formed. These will then be in the ratio by weight of $n: (n - 1): (n - 2): \dots: 2:1$, and the total weight (w_{total}) of such a series of oligomers is the sum of an arithmetic progression, i.e.,

$$w_{\text{total}} = n/2[n + 1]w,$$

TABLE III
CALCULATED YIELDS OF MALTODEXTRINS

Expt. ^a	Theoretical assumption	% by weight						Higher sugars
		G ₁	G ₂	G ₃	G ₄	G ₅	G ₆	
1a	Experimental	1.8	8.5	9.6	6.2	5.5	14.3	54.0
	(i) G ₅ stable ^b	2.1	4.2	6.3	8.5	10.6	68.3	
	(ii) G ₆ stable ^c	2.2	4.4	6.6	8.8	11.0	13.2	53.8
	(iii) Random ^d	4.0	6.4	7.7	8.2	8.2	7.9	57.6
2	Experimental	4.7	13.2	10.7	7.3	6.7	27.8	29.5
	(i) G ₅ stable ^b	2.8	5.7	8.6	11.4	14.3	57.2	
	(ii) G ₆ stable ^c	3.4	6.7	10.1	13.4	16.8	20.2	29.4
	(iii) Random ^d	11.1	14.8	14.8	13.2	11.0	8.8	26.3

^a As in Table II.^b Calculated from Eq. (2) with $n = 5$; $i = 0, 1 \dots 4$; $x = 0.32$ for expt. 1a; $x = 0.43$ for expt. 2.^c Calculated from Eq. (2) with $n = 6$; $i = 0, 1 \dots 5$; $x = 0.46$ for expt. 1a; $x = 0.71$ for expt. 2.^d Calculated from Kuhn's theory (8) with a value of s chosen so that the yield of G₇ and >G₇ was comparable with that found experimentally.

where w is the weight of monomer unit. Thus the fraction by weight of total degraded material existing as an $(n - i)$ mer is

$$(n - i)/[n(n + 1)] = 2(n - i)/n(n + 1). \quad (1)$$

This relation gives the theoretical yields of oligomers when a polymer is degraded *completely* to oligomers of size n and smaller. However, in our experiments, the mixture of oligomers was analyzed before degradation was complete, and hence if a fraction x of total polymer has been degraded, then fractional yields of oligomers can be calculated from

$$W_{n-i} = \frac{2(n - i)}{n(n + 1)} x. \quad (2)$$

In Table III the experimental yields of oligosaccharides are compared with theoretical yields calculated on the assumption that degradation is random but that (i) G₅ and smaller oligomers are effectively resistant, (ii) G₆ and smaller oligomers are effectively resistant, and (iii) no oligomers are effectively resistant to α -amylolysis.

In the application of Kuhn's theory (8) to calculate yields for assumption (iii), a value for the degree of scission, s , was arbitrarily

chosen so that the the total yield of oligomers greater than G₆ approximated to that found experimentally. Now it can be easily shown that for any other value of s , there is a correspondingly similar theoretical distribution of the oligomer yields, i.e., there is a *continuous* increase in amount up to a certain oligomer followed by a *continuous* decrease thereafter; an experimental determination of s is therefore unnecessary to demonstrate this point.

It can be seen that our experimental yields do not follow such a pattern, and so, notwithstanding the evidence from the viscosity experiments, the α -amylolysis cannot be random at the later stages.

However, the qualitative experiments on production of oligosaccharides suggest that G₆ itself is relatively stable, and our experiments on other plant α -amylases have confirmed this. On this basis, and the fact that glucose is not too readily produced from the α -amylolysis of maltodextrins, we would suggest that, in oligosaccharides, (i) the five bonds adjacent to the nonreducing endgroup, and (ii) the bond next to the reducing group are all *less readily* susceptible to degradation. A similar conclusion has been arrived at by Bird and Hopkins (9).

Elsewhere (10), we have developed this

hypothesis and have shown that it will account satisfactorily for relative ease with which individual maltodextrins are degraded. Further, it is suggested that the two apparent stages in the α -amylolysis of amylose are not due to different reactions but are merely a result of the difference in affinity of the enzyme for large and small molecules.

It is to be noted that the action pattern of this plant α -amylase differs from that described for a bacterial α -amylase (11).

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The α -Amylolysis of Starch^{*})

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Introduction

α -Amylases have formed the subject of extensive investigations and their general properties are well-established. However, it now seems very likely that the specific mode of action of any α -amylase depends on the source from which the enzyme is isolated. In particular, there is evidence that the action-pattern of α -amylases from mammalian-, plant-, fungal- and bacterial-sources may differ, although enzymes from any particular source, e.g. cereals, might be expected to be similar. Investigations along these lines are limited. Rather surprisingly, with the exception of the enzyme from malted-barley, plant α -amylases have been little examined.

We have recently started a study of α -amylases from plant sources and have evolved a general isolation procedure which will readily give good purification. This has enabled us to investigate the properties and specific action-pattern of some of these enzymes, and the results of our investigations are considered in this paper.

Sources of the α -amylases

The first α -amylase that we investigated was the "Z-enzyme" of the soya-bean. This enzyme has been the subject of some controversy. It was first shown to exist as a contaminant of impure soya-bean β -amylase when the mixture gave complete conversion of any amylose sample into maltose, whilst crystalline sweet potato β -amylase gave incomplete conversion (1, 2). Later work on this enzyme yielded conflicting results (3–7), but the general conclusion was that "Z-enzyme" was specific for removing the barrier to β -amylase in amylose. Although the preparation of "Z-enzyme" has been described (8), this work has not been repeated, and in our earlier studies we had to inhibit the β -amylase to obtain the "Z-enzyme" (9). Our results then showed conclusively that "Z-enzyme" exerted a random hydrolytic attack on amylose by the observed rapid fall in viscosity of the amylose/Z-enzyme digests. The enzyme also attacked linear amylose (i.e. amylose which was completely degraded into maltose by β -amylase) and the postulate that "Z-enzyme" specifically removed the barrier to β -amylase action was shown to be incorrect. We found that "Z-enzyme" had the properties of an α -amylase, but although amylopectin was degraded, glycogen appeared not to be so (9).

In view of the general α -amylolytic character of "Z-enzyme", we decided to attempt an isolation procedure from the soya-bean based on this property. This preparation was successful and hence we have been able to examine the properties of the enzyme and its action-pattern in detail (10).

An analogous problem existed in the case of barley where again non-crystalline preparations of β -amylase are contaminated by an enzyme which has the characteristics of soya-bean Z-enzyme. We have isolated, therefore, the α -amylolytic component of barley, and examined its properties (11).

It has to be stressed that the amount of α -amylase in the soya-bean and barley is so small that conventional tests for α -amylolytic activity are usually negative (12). This makes the isolation of the enzymes more difficult.

The successful isolation of barley α -amylase made it of interest to compare its properties with those of malted barley α -amylase. For this purpose, we malted a portion of the sample of barley from which the α -amylase had been isolated. We were thus able to compare the two enzymes in detail (11).

α -Amylase has also been isolated from the broad-bean (*Vicia faba*, Linn.), where it exists along with many other carbohydrases. This α -amylase has been isolated and its properties examined for the first time (13).

Measurement of α -amylase activity

In the initial stages of the preparation of α -amylase from barley and soya-bean, a method of estimating α -amylolytic activity was required which would be effective in the presence of contaminating β -amylase. Many methods of estimation are described in the literature, but most of them do not distinguish between the activities of α - and β -amylases. For example, the method of FISCHER and STEIN (14) entails the estimation of reducing power, and hence traces of β -amylase would invalidate the results. The method of HULTIN (15), which involves measuring the fall in viscosity of enzyme-polysaccharide digests, will also be suspect in the presence of β -amylase. Several methods utilize the property of α -amylase to decrease the colour of a starch-iodine complex (16), but the presence of β -amylase will also affect these.

The use of amylopectin β -limit dextrin, however, as the substrate for the enzyme will not be affected by the concurrent presence of β -amylase and in the early stages any reduction in the ability to stain with iodine will be a true measure of α -amylolytic scission. Amylopectin β -limit dextrin is not completely ideal as in the presence of excess β -amylase, α -amylolytic attack will be followed by β -amylolysis. However, because the dextrin molecule is fairly compact and the lengths of chain available to the β -amylase after α -amylase attack are small, the effect of β -amylolysis was found to be negligible.

However, even with the use of this limit-dextrin, the measurement of α -amylolytic activity by changes in the colour of the iodine-complex is not simple. The absorption value, I , falls off non-linearly with time, t , and so a function of the type $(I_0 - I_t)/[I_0(t_1 - t_0)]$ is not satisfactory. We have also found that taking the time of half-life (i.e. assuming an exponential fall in absorption value) is not accurate, and measurements

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of the initial rate of decrease of the iodine-staining power are not reproducible. The method that was found to be satisfactory was the modification by BRIGGS (17) of the SANDSTEDT, KNEEN and BLISH assay (18), where activity is expressed as the reciprocal of the time taken to decrease the dextrin-iodine stain by a given amount. A standard graph was constructed (in our experiments, by using different dilutions of

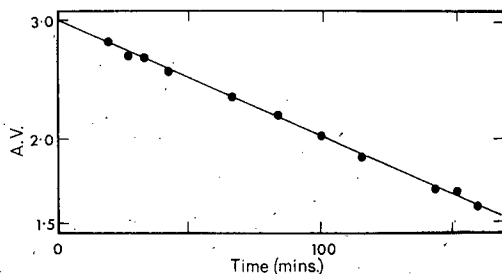


Fig. 1. Standard graph for α -amylase activity; "corrected absorption value" (A.V.) versus "relative time." (17).

salivary α -amylase) in which the time required for the absorption value to fall by an arbitrary amount (from a colorimeter reading of 3.00–2.00) was taken as 100 mins. The typical standard graph used is shown in Fig. 1, and enzyme activities were expressed as the apparent activity in these iodine dextrin-colour units per mg. of protein in 1 ml. of digest.

Purification of plant α -amylases

The method that has been found to be generally applicable to the purification of plant α -amylases can be summarized as follows:

1. initial aqueous extraction of flour in the presence of Ca^{2+} to stabilize the enzyme.
2. fractionation with acetone at -5°C
3. heat treatment at 70°C in presence of Ca^{2+} to remove contaminative β -amylase (if necessary).
4. re-fractionation with acetone at -5°C
5. formation of glycogen- α -amylase complex following the procedure of SCHRAMM (19).

This procedure resulted in an increase in specific activity of the α -amylase some 150–700 times that of the original extract. The purification achieved at each stage in purification of the broad-bean enzyme is shown in Fig. 2; as with most of the other samples, the stage resulting in the largest increase in specific activity was that involving the formation of the glycogen complex. However, it has to be noted that glycogen-complex formation is dependent on the amount of α -amylase present in the preparation, and in the case of the barley enzyme, the concentration was so small that complex-formation did not occur. All the α -amylase preparations were free from the presence of other carbohydrases and phosphatases.

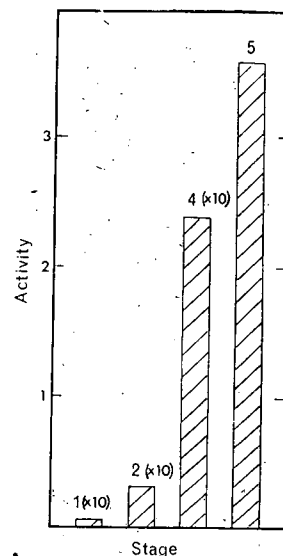


Fig. 2. Purification of broad-bean α -amylase; activity in arbitrary units. For significance of stages see Text.

Properties of the α -amylases

Table 1 shows the general properties of the four α -amylases. The pH of optimum activity is very comparable, and the pH/activity curves (see Fig. 3) were independent of the substrate concentration over the range studied for the soya- and broad-bean enzymes. On this basis, these curves have been analysed by considering them as being due to the ionization of two reactive groups in the enzyme in order to determine the pK -values (20–22).

In the case of the soya-bean enzyme, values of $pK_a = 8.15$ and $pK_b = 4.3$ were found. The group with $pK_b = 4.3$ is most likely a carboxyl group. A pK_a -value of 8.15 corresponds to an ammonium group, but there is the possibility that some anion may have displaced the pH-activity curve (23), in which case an imidazolium-group may be involved. By use of specific inhibitor reagents and the formation of derivatives

Table 1
Comparison of the general properties of plant α -amylases¹⁾

Property	Soya-bean	Barley	Malted barley	Broad-bean
Optimum pH	6.0	5.5	5.5	5.6
pK_a	8.15	n. d.	n. d.	7.1
pK_b	4.3	n. d.	n. d.	4.1
% inactivation by incubation at pH 3.6	100	100	100	100
Optimum temperature ($^\circ\text{C}$)	55	47.5	47.5	45
Apparent energy of activation (kcal)				
at 9°C	14	13	13	14
at 25°C	6	13	13	5
at optimum temperature	0	0	0	0
% inactivation by 10^{-3} M Hg^{2+}	100	100	100	100
% inactivation by $10^{-3}\text{ M ascorbic acid}$	100	n. d.	93	n. d.
% inactivation by 10^{-3} M CN^-	0	0	0	0
% inactivation by EDTA ²⁾ and trypsin	100	100	100	100
% activation by Ca^{2+}	0	0	0	0
% activation by Cl^-	0	0	0	0

¹⁾ n. d. = not determined

²⁾ EDTA = ethylenediaminetetraacetate

(10), we showed that this grouping was, in fact, very likely to be involved in the active-centre.

Values of $pK_a = 7.1$ and $pK_b = 4.1$ were found for the broad-bean α -amylase, and again it appeared that these corresponded to a carboxyl- and an imidazolium-group (13).

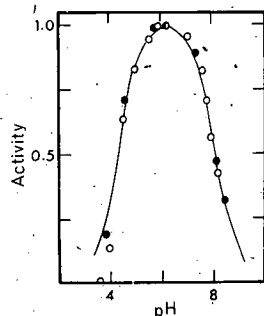


Fig. 3. pH/activity curve for soya-bean α -amylase (10). Activity is expressed relative to that at optimum pH; the closed- and opened-points represent different concentrations of substrate.

One of the features initially considered to be a characteristic of "Z-enzyme" was the fact that it was inhibited at pH 3.6. However, as Table 1 shows, it would appear that this is true of many plant α -amylases and not unique to the soya-bean enzyme.

The temperature of optimum activity was ca 45 °C, except for the soya-bean α -amylase which was appreciably higher. However, the apparent energies of activation at various temperatures were very comparable.

All the enzymes required the presence of calcium ions for stability, but an excess of these ions apparently did not cause activation. It was also found that an excess of chloride ion had no effect. In the absence of calcium ions, the α -amylases were made susceptible to protease attack (e.g. by trypsin), and all the enzymes were irreversibly denatured by the joint action of sodium ethylenediaminetetraacetate (EDTA) and trypsin. Inhibition by the action of EDTA alone was not complete, and furthermore was reversible to some extent on the addition of calcium ions. The α -amylases were all inhibited by the presence of 10^{-3} M mercuric chloride and 10^{-3} M ascorbic acid, but were unaffected by 10^{-3} M potassium cyanide.

All the α -amylases hydrolysed amylose, amylopectin, amylopectin β -limit dextrin, glycogen and glycogen β -limit dextrin. It is to be noted that in the case of the soya-bean enzyme, this conclusion is different from our earlier observations (9). Typical action-patterns are shown in Fig. 4a and 4b. The α -amylolysis of amylose is characterized by occurring in two apparent stages (Fig. 4b); there is an initial very rapid increase in reducing-power followed by a much slower one, the transition occurring at the "achroic limit" of the digest. However, it has to be noted that the "achroic limit" is an arbitrary concept as discussed below.

Action-pattern of the α -amylases

The detailed action-pattern of the four α -amylases was investigated by using linear amylose (24) as the substrate. This substrate was chosen to eliminate the production of branched malto-dextrin products. Various aspects of the α -amylolysis shown in Fig. 4b. were

considered: (1) the kinetics of the initial reaction (stage 1) were followed viscometrically, and (2) the production of maltodextrins at the "achroic limit" (stage 2) and beyond (stage 3) was investigated by both qualitative and quantitative paper-chromatography.

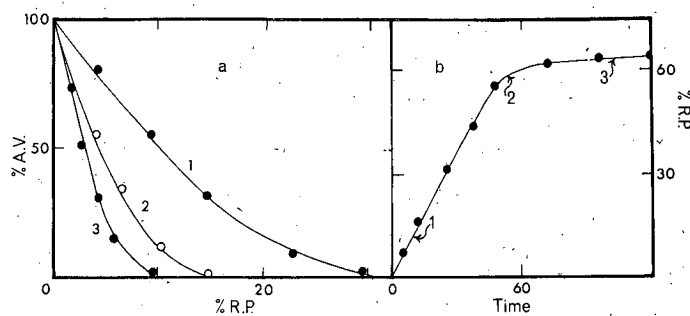


Fig. 4. (a) % of original absorption value (A. V.) versus apparent reducing-power (R. P.) for the barley α -amylolysis (11) of

1. amylose
2. amylopectin
3. amylopectin β -limit dextrin.

(b) apparent reducing-power (R.P.) versus time (in hours) for the α -amylolysis of amylose showing (1) initial hydrolysis stage, (2) "achroic limit", and (3) later stage in degradation.

Kinetic investigations of the initial reaction

In this stage of the α -amylolysis, the amylose molecules are being rapidly degraded in molecular size as shown by the loss in iodine-staining ability and the increase in reducing-power. However, there is some controversy as to whether or not this degradation is a random hydrolytic process. The kinetics of the degradation have therefore to be studied, but this involves both theoretical and experimental difficulties.

To follow a depolymerization process of this type, a counting of the number of polymer molecules at different stages is required. This necessitates measuring a colligative property, and of these, only osmotic pressure is suitable for polymers. However, osmotic pressure measurements are extremely difficult to carry out, and the method is insensitive to small degrees of depolymerization. In contrast, viscosity measurements—although not yielding a number-average quantity—are particularly sensitive for measuring the effect of the scission of only a few bonds per polymer molecule, i.e. the breaking of only one bond will, on average, halve the viscosity. Furthermore, viscosity measurements can be used to give values of degree of polymerization, DP , by use of the well-known relation that

$$[\eta] = K (DP)^\alpha,$$

where $[\eta]$ is the limiting viscosity number, and K and α are constants for a given polymer/solvent system.

Now for a first or zero-order depolymerization process the rate is proportional to $1/DP$. We have used the method of VINK (25) to obtain this function from measurements of the specific viscosity, η_{sp} , of the amylose/ α -amylase digest at various times. VINK (25) has shown that for a random depolymerization process, the graph of $1/DP$ versus time is linear, whereas for a non-random process this graph is non-linear.

For all the α -amylases, the function $1/DP$ versus time evaluated from measurements of η_{sp} was linear as shown in the typical result in Fig. 5. From these

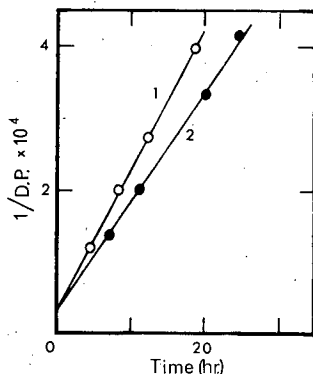


Fig. 5. The graph of $1/DP$ versus time for amylose (10) under the action of
1, soya-bean α -amylase,
2, 0.5 M hydrochloric acid.

results, it was concluded that the initial stages in the α -amylolytic process involved a purely random scission of α -1:4-glycosidic bonds. This conclusion was substantiated when the results for the acid-catalysed depolymerization of the amylose sample—a known random process—were found to be the same (cf. Fig. 5). It has to be stressed, however, that although very large changes in η_{sp} occurred in these experiments, i.e. corresponding to 20 or more bonds per initial amylose molecule being broken on average, the percentage of bonds broken was less than one.

Production of maltodextrins at the achroic limit

The results of a typical qualitative paper-chromatographic examination of an amylose/ α -amylase digest are shown in Table 2. The general pattern of maltodextrin production that has emerged from this work is that at the "achroic limit", maltodextrins greater than G_6 predominate, but all the lower sugars—including glucose—are also present. Then as the α -amylolysis proceeds, all the maltodextrins G_1 to G_8 increase in amount before the higher ones begin to decrease.

Table 2

Relative amounts¹⁾ of maltodextrins present after treating amylose with malted barley α -amylase (11)

Malto-dextrin ²⁾	Incubation time (hr.)						
	1/2	1	2	12	50	96	120
G_1	+	+	2 +	3 +	5 +	5 +	5 +
G_2	+	2 +	3 +	4 +	5 +	5 +	5 +
G_3	+	2 +	3 +	3 +	3 +	4 +	4 +
G_4	+	2 +	3 +	4 +	5 +	5 +	5 +
G_5	+	2 +	2 +	3 +	4 +	4 +	4 +
G_6	2 +	3 +	4 +	5 +	4 +	3 +	3 +
G_7	2 +	4 +	5 +	2 +	+	0	0
G_8	4 +	4 +	+	0	0	0	0
$> G_8$	5 +	2 +	0	0	0	0	0

¹⁾ The number of + signs indicates the relative amount of maltodextrin; amounts can only be compared horizontally not vertically.

²⁾ G_1 = glucose; G_2 = maltose; G_3 = maltotriose; etc.

The arbitrary nature of the "achroic limit" is shown by the fact that, if the digest at this point is concentrated (say) 100-fold, it stains blue with iodine. As usually determined, the "achroic limit" depends primarily on the enzyme/substrate ratio. Our experiments show that a digest is truly achroic only when no maltodextrins greater than G_7 are present.

The fact that certain of the lower maltodextrins tended to persist in the digests suggested that there might be a reduction in the susceptibility of these materials to α -amylolysis. Individual sugars were therefore incubated with the α -amylases. Table 3 shows the general trend that became apparent from these experi-

Table 3

Products from the action of α -amylase on maltodextrins

Malto-dextrins	Major products	Minor products	Trace products
G_4	G_2		$G_1 + G_3$
G_5	$G_3 + G_2$		$G_1 + G_4$
G_6	$G_4 + G_2, G_3$		$G_1 + G_5$
G_7	$G_1 + G_6$	$G_4 + G_3$	$G_2 + G_5$
G_8	$G_2 + G_6$	$G_7 + G_1, G_4$	$G_5 + G_3$
G_9	$G_2 + G_7$	$G_6 + G_3, G_1 + G_8$	$G_5 + G_4$
G_{10}	$G_2 + G_8, G_3 + G_7$	$G_4 + G_6$	$G_5, G_1 + G_9$

ments; there is not the full range of products that would be expected on random degradation, and furthermore the rates of hydrolysis were $G_4 < G_5 < G_6 < G_7 < G_8$.

Two noticeable features of these results are (1) the lack of glucose as a major product (except from G_7), and (2) the persistent lack of G_5 as a product from the hydrolysis of all maltodextrins $> G_5$.

The significance of these results is discussed below.

Quantitative estimations of maltodextrin formation

In the case of the soya- and broad-bean α -amylases, the formation of maltodextrins at the "achroic limit" and later stages in the digest was investigated by quantitative paper-chromatography. Results are shown in Table 4.

These experimental values have been compared with those calculated on the basis of KUHN's theory of random degradation (26). It can be seen (Table 4) that the experimental values are radically different from the theoretical ones, and so—notwithstanding the evidence from the viscosity experiments above—the α -amylolysis is not likely to be random at this stage.

The results of the experiments on the α -amylolysis of the maltodextrins suggested that G_6 , G_5 and $< G_5$ were more resistant to hydrolysis than G_7 and $> G_7$. Theoretical yields of maltodextrins were therefore calculated from the theory of PAINTER (27) assuming random hydrolysis of the amylose polymer but also that certain maltodextrins were resistant to further α -amylolysis. Table 4 shows the theoretical yields calculated on this basis with (1) G_5 and $< G_5$ stable, and (2) G_6 and $< G_6$ stable. Again, although slightly better agreement with the experimental values is obtained when G_6 is regarded as being effectively resistant, for both cases, more G_2 and G_6 and less G_4 and G_5 than predicted are found experimentally. The α -amylolysis cannot therefore be completely random.

Table 4
Experimental and theoretical yields of maltodextrins from amylose

Enzyme	Yield *)	% by weight of						
		G ₁	G ₂	G ₃	G ₄	G ₅	G ₆	> G ₆
Soya-bean	E	7	13	11	7	6	31	25
	R	11.1	14.8	14.8	13.2	11.0	8.8	26.3
	R ₅	3.0	6.0	9.0	12.0	15.0	55	
	R ₆	3.6	7.1	10.7	14.3	17.9	21.4	25
Broad-bean	E	4.7	13.2	10.7	7.3	6.7	27.8	29.5
	R	11.1	14.8	14.8	13.2	11.0	8.8	26.3
	R ₅	2.8	5.7	8.6	11.4	14.3	57.2	
	R ₆	3.4	6.7	10.1	13.4	16.8	20.2	29.4

*) E = Experimental.

R = Theoretical random degradation.

R₅ = Theoretical random degradation with G₅ resistant

R₆ = Theoretical random degradation with G₆ resistant

Now in order to explain the high yield of G₆, it might be postulated that the α -amylase is unable to catalyse the hydrolysis of the five bonds nearest the non-reducing end of an amylose molecule. A consideration of the yields of the possible breakdown-products then shows that after an attack on (say) G₁₂, the products would consist of a high yield of G₆ and equal amounts by weight of the smaller sugars. If the additional restriction is made that the bond adjacent to the reducing-group is also resistant—in view of the lack of glucose produced from the oligosaccharides—then it can be shown that there will be a high yield of G₇ and no G₁ as follows:

Maltodextrin	G ₁	G ₂	G ₃	G ₄	G ₅	G ₆	G ₇
% yield	0	10.5	9.2	8.8	8.3	41.6	21.6

It is obvious that neither of these two schemes accounts accurately for the observed yields, but that the second is more satisfactory as it shows the tendency to form less G₄ and G₅ and more G₂ and G₆.

It is perhaps reasonable, therefore, to consider that an intermediate scheme might explain the results, i.e. that some bonds in a linear maltodextrin are more resistant to α -amylolytic attack than others. It seems likely that these particular bonds are (1) the one adjacent to the reducing-group and (2) the five bonds adjacent to the non-reducing end. This hypothesis is similar to that proposed by HOPKINS and BIRD (28).

Hypothetical scheme for enzyme action

A hypothetical scheme for the α -amylase action based on the above is shown in Fig. 6. It is considered that the active centre of the enzyme—containing the pH-dependent nucleophilic and electrophilic groups which cause catalytic scission of the glycosidic bond—will bind eight glucose units under the most favourable conditions, i.e. six on one side and two on the other of the scission point.

A consideration of the mode of breakdown of the maltodextrins suggests that there may be preferential ease of binding of (1) non-reducing end-groups at site N and (2) reducing end-groups at site R. In addition, the ease of combination of enzyme with substrate is reduced at positions, X, as shown by the relatively low

formation of G₁ and G₅. The latter suggests that there may be some steric factor involving the C1—OH or the C4—OH of the glucose units, respectively, at these points.

Fig. 7a and b shows the most favourable and unfavourable degradation products that might be expected to arise from oligosaccharides on this basis. The scheme accounts satisfactorily for the experimental observations with the exception of G₇; for this maltodextrin the favourable effect of site N overrides the adverse effect of X₂.

The greater stability of G₆ and < G₆ to α -amylolysis is probably due to the lower statistical probability of a substrate of this size interacting with the active centre in such a way that glycosidic-cleavage occurs.

In the case of the α -amylolysis of amylose, although the viscosity-results from the early stages of hydrolysis indicate a random enzymic action, whilst the chromatography indicates a non-random attack, these results are not necessarily inconsistent with the above scheme.

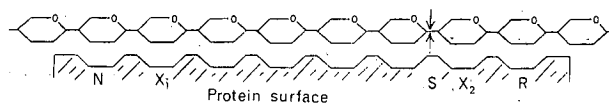


Fig. 6. Hypothetical active site for α -amylase


S = scission point

N = preferential binding site for a non-reducing end-group

R = preferential binding site for a reducing end-group

X₁ = specific unfavourable binding site for a non-reducing end-group

X₂ = specific unfavourable binding site for a reducing end-group

 = glucose ring in an amylose-chain

Because the enzyme will not hydrolyse bonds near the ends of molecules as easily as others, at all stages, the α -amylolysis cannot be completely random although in practice the initial stages will appear to be so.

For example, if an amylose molecule of DP = 3000 is considered, then there will be only ca 6 bonds at the ends which will be resistant to enzymic attack, leaving 2993 bonds which may be attacked easily and randomly. In the initial stages, therefore, the process will appear to be random. As hydrolysis proceeds and the substrate molecules become smaller, the ratio of non-resistant to resistant bonds decreases, and so the ease of attack on the molecule decreases. As a result, the non-random nature of the process becomes more important, with a consequent decrease in the rate of bond scission. This accounts for the very slow increase in reducing power after the apparent "achroic limit".

In the final stage of the amylolysis, when only G₈ and smaller maltodextrins are present, there are few non-resistant bonds in the substrate molecules and the reaction is very slow and non-random.

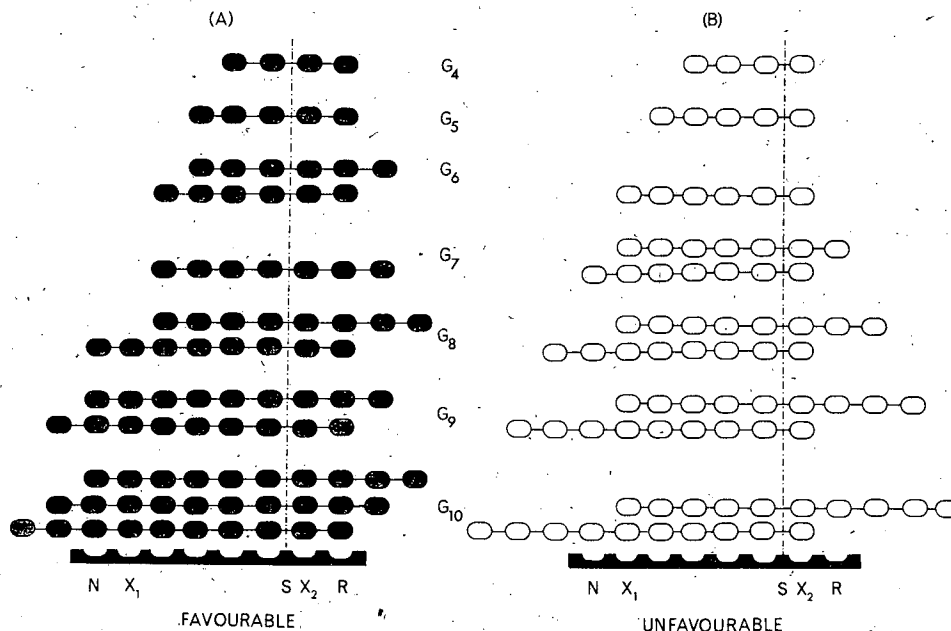


Fig. 7. Interaction of maltodextrins G_4 — G_{10} with hypothetical active site showing the theoretical favourable hydrolytic products (A), and the unfavourable hydrolytic products (B).

It seems most likely then that the two apparent stages in the α -amylolysis of amylose are not due to different and separate reactions, but merely reflect a difference in the affinity of the enzyme for large and small substrate molecules.

Finally, it should be noted that although the action-pattern of these two plant α -amylases is very similar, it is appreciably different from that of the α -amylase from *B. Subtilis* (29). This aspect will be dealt with later.

Conclusions

A consideration of the general properties and action-pattern of the four α -amylases suggests that there is little to distinguish the soya-bean enzyme from the others. Furthermore, the enzyme from the barley and the corresponding malted barley are also similar, and our general conclusions are that the α -amylase present in the barley is a dormant form which becomes active on germination without a significant change in properties.

Acknowledgements

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Summary

As part of a general study of plant α -amylases, the enzymes present in the soya-bean, barley and malted barley, and the broad-bean have been investigated.

A successful method for measuring α -amylolytic activity in the presence of β -amylase is outlined, as well as a general procedure for purifying plant α -amylases free from other carbohydrases.

The properties of the purified enzymes have been described, and the α -amylolytic degradation of amylose investigated in detail. Viscometric measurements have shown that the initial stage of the degradation is apparently a random hydrolytic process. The production of maltodextrins at the achroic limit has been shown by

paper-chromatographic analysis to occur in a non-random manner; some small maltodextrins being resistant to α -amylolytic attack. Quantitative aspects of the production of maltodextrins are described, and a hypothesis presented to explain the action-pattern of these plant α -amylases.

Zusammenfassung

Im Rahmen einer allgemeinen Untersuchung von pflanzlichen α -Amylasen wurden die in Sojabohnen, Gerste, gemälzter Gerste und Pferdebohnen enthaltenen Enzyme analysiert.

Es wird eine erfolgreiche Methode zur Messung der α -amylolytischen Aktivität in Gegenwart von β -Amylase sowie eine allgemeine Verfahrensweise für die Reinigung von pflanzlichen α -Amylasen von anderen Carbohydrasen dargelegt.

Die Eigenschaften der gereinigten Enzyme werden beschrieben und der α -amylolytische Abbau von Amylose eingehend untersucht. Viskometrische Messungen haben ergeben, daß die erste Phase des Abbaus wahrscheinlich einen willkürlichen Hydrolyseprozeß darstellt. Mit Hilfe der Papier-Chromatographie wurde gezeigt, daß die Entstehung von Maltodextrinen an der „achroic limit“¹⁾ nicht willkürlich ist; einige kleine Maltodextrine²⁾ sind gegen α -amylolytische Einwirkung beständig. Die quantitativen Aspekte der Bildung von Maltodextrinen werden beleuchtet, und es wird eine Hypothese aufgestellt, um die Wirkungsweise dieser pflanzlichen α -Amylasen zu erklären.

Résumé

Dans le cadre d'une analyse générale des α -amylases végétales, on a analysé les enzymes contenus dans les sojas, l'orge, l'orge maltée et les fèves de marais.

On explique une méthode fructueuse capable de mesurer l'activité α -amylolytique en présence de la β -amylase. En outre on donne une méthode générale servant à dépur

¹⁾ Abbaufstufe der Stärke, von der ab keine Blaufärbung mit Jod mehr auftritt

²⁾ small maltodextrins: unverzweigte nieder-molekulare Dextrine

les α -amylases végétales des autres carbohydrases accom-pagnantes.

On décrit les propriétés des enzymes dépurés et on donne une analyse détaillée de la dégradation α -amylolytique de l'amylose. Des mesures viscométriques ont montré que le point initial de la dégradation est apparemment dû à un procès hydrolytique arbitraire. Une analyse par chromatographie sur papier a montré que la production de maltodextrines à la limite achroïque ne se produit pas d'une façon arbitraire. En effet quelques petites maltodextrines, résistent à une attaque α -amylolytique. On décrit des aspects quantitatifs de la production de maltodextrine, et on établit une hypothèse pour expliquer la manière d'action des α -amylases végétales.

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ABSTRACT OF THESIS

Name of Candidate..... Elizabeth Ann Milne
Address..... 65 Findlay Gardens, Edinburgh, 7.
Degree..... Doctor of Philosophy..... Date..... May, 1966.
Title of Thesis..... Studies on Starch-Degrading Enzymes.

A brief account is given of the actions of the more important starch-degrading enzymes. The general experimental techniques used in this study are outlined, and a method for measuring α -amylolytic activity in the presence of β -amylase is described.

α -Amylases have been isolated from soya- and broad-beans, oats, rye, wheat, germinated barley and germinated wheat. These amylases have been purified free from other starch-metabolizing enzymes by a procedure involving acetone fractionation and heat-treatment in the presence of an excess of calcium ions. The method used to prepare the bean and germinated cereal α -amylases includes the formation of an insoluble glycogen-enzyme complex.

The effect of various salts and of temperature on the activity of the α -amylases from oats, rye and wheat has been examined. Viscometric techniques have been used to study the activity of these cereal enzymes at pH 3.6 and in the presence of ethylene diamine tetraacetate and trypsin. Variation of amylolytic activity with pH and with modification of the enzymes has enabled the nature of the amino acids at the catalytically active centres of the cereal and bean amylases to be investigated. These amino acids are thought to contain free carboxyl, imidazolium and amino groups, but not sulphydryl groupings.

The molecular sizes of the α -amylases of germinated and dormant wheat and germinated barley have been compared by gel-filtration techniques. The results indicate that the three enzymes are of similar molecular size, i.e. no large change in molecular weight of a cereal α -amylase takes place on germination of the cereal.

The action-patterns of several α -amylases on linear substrates have been investigated. The kinetics of the initial stage of the α -amylolysis of amylose by the enzymes from oats, rye and wheat have been studied viscometrically. The results indicate that the process is random. However, paper-chromatographic investigations of the production of maltodextrins from amylose and the hydrolysis of small oligosaccharides have shown that, at later stages, non-random attack predominates. This has been confirmed by determining the yields of maltodextrins produced from amylose after the achroic limit. Plant, bacterial and mammalian α -amylases have been compared, and four types of action-pattern have been found, corresponding to the enzymes from beans, cereals,

B. subtilis and porcine pancreas. Little change appears to take place in the action of a cereal α -amylase on germination of the cereal.

Theories for the action-patterns of the different types of α -amylases have been proposed to account for the experimental results. The enzymes are thought to act in a non-random manner on α -1:4 bonds near the chain-ends of substrate molecules, but hydrolyse randomly all other α -1:4 bonds. Hypothetical schemes are presented for the active centres of bean, cereal, bacterial and mammalian α -amylases.